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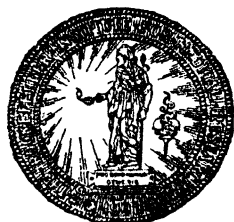
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II. CATABOLISM. CHEMICAL CHANGES IN FERTILE EGGS DURING INCUBATION. SELECTION OF STANDARD CONDITIONS.*

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INTRODUCTION.

Kirchoff (1) stated that the object of mechanical science was "to describe completely and in the simplest manner the motions which occur in nature," and so, from the standpoint of energetics, life may be viewed as a mechanism and described in terms of the absorption and elimination of energy. In the absence of external work, the developed energy resolves itself into heat, and as such may accordingly be estimated directly or indirectly, so it seems, by measuring the exchange of the respiratory gases. For a proper understanding of these processes one must know the quantity and quality of material ingested in a given unit of time, and what part of the absorbed potential energy is retained and what part converted and dissipated as heat. The experiments to be presented in this paper deal with the latter factor since they involve determinations of the amount of carbon dioxide eliminated by embryos of successive incubation ages. These tests were supplemented by chemical analyses before and after incubation. The chemical analyses were to serve also as a preliminary to a subsequent study of the changes in the constitution of the embryo with age, so that a comparison might be made of the concentrations of the more important substances inside and outside the embryo.

It is important to know the amount of (1) fuel (carbohydrate, protein, and fat) burned in embryonic metabolism, (2) water lost by

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* An article of an introductory nature dealing with the general point of view from which these experiments were undertaken will be published elsewhere.

evaporation, and (3) solid substance, if any, contributed to the developing embryo by the egg shell. We assume that the weight of all other non-oxidizable substances within the egg remains constant throughout incubation.

Preparatory to these and subsequent experiments it was necessary to establish suitable incubation conditions, the maintenance of which would increase the probability that development proceeded at approximately the same rate in each egg. With this object a few observations have been made upon the atmospheric temperature and humidity as variables modifying the constitution of the whole egg during incubation.

The Source and Control of the Experimental Material.

Unless it is stated to the contrary the management of the fertile egg as now described applies to all subsequent experiments of this series. The eggs are those of White Leghorn hens 9 to 18 months of age raised and kept on the same farm. They are collected several times a day, refrigerated at about 55°F. during the night, shipped the next morning and delivered cold at the laboratory the following day. Approximately 2 days after they are laid they are incubated in the laboratory.

There are numerous variable factors which can modify the constitution of the egg as well as the rate of development of the blastoderm (2). Investigations at agricultural experimental stations have revealed that the amount of yolk, albumin, and water, as well as the thickness of the shell, vary according to the season, diet, and general condition of the bird (3). Variations in the relative proportion of these substances may also occur as a result of individual or chance happenings, such as the velocity of the egg's passage down the oviduct whence the albumin is derived, the time intervening between laying and collecting, the humidity and temperature of the surrounding atmosphere after laying and during transportation. As the initiation of ontogenesis by fertilization occurs in the upper reaches of the oviduct and as the process of egg formation in the hen usually takes from 18 to 30 hours, the development of the blastoderm with differentiation of the three germ layers has already taken place by the time the egg is laid. Variations then in the duration of the egg's stay within the

reproductive organs of the hen so affects development that no two embryos will be exactly the same age at the time of laying. Poultry-men recognize what are called body-heated eggs, that is to say, eggs held in the body of the fowl for 2 or more days. Since temperature conditions are suitable, development will be found to have progressed further than usual in such eggs. If, after laying, the temperature is ever raised as high as 72°F. it is said that growth of the blastoderm will commence and proceed slowly. Likewise, if a hen sits on the egg for a time after laying, the stage of development at the commence-ment of incubation will be more advanced than the average. These, then, are some of the factors, which in our experiments were more or less variable. Their importance could only be minimized by the accumulation of sufficient data, that is to say, by the statistical method.

In a few of the subsequent experiments the eggs were kept in a Lo-Glo incubator at about 39°C. Once a day they were taken out into the laboratory and rolled. Later, when a constant temperature room was built, a water-jacketed copper box provided with a constant inlet of warm fresh air was used as an incubator. The maximum variation of temperature within the box was ± 0.4.[1] The eggs were turned once or twice a day and weighed at less frequent intervals. All manipulations were done in the room where the humidity was less than in the box, but the temperature was approximately constant at 38.8° ± 1.0°C.[1]

Weight and Surface Area of Eggs.

The average weight of over 500 eggs before incubation was found to be 57.8 gm. It is stated in the literature that during incubation there is a loss of weight most of which can be accounted for by the evaporation of water. The extent of the loss was usually very vari-able, but there were found no analyses of the factors which determined it. As the amount of evaporation and gaseous exchange in the egg must be a function of its surface rather than of its mass, it was con-sidered advantageous to find a convenient method for measuring the surface area. Three simple indices were tried for each of twenty-seven

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[1] ± 0.4 and ± 1.0 refer to the maximum range.

eggs: (1) the projected surface. To obtain this a piece of apparatus was constructed to hold the egg firmly in position resting on a flat sheet of paper. The greatest circumference of the egg was then drawn with a pencil held perpendicularly in a block and the enclosed area representing the largest cross-section of the egg was measured with a planimeter; (2) the product of the maximum length and width; and (3) the two-thirds power of the weight $(W^{\frac{2}{3}})$ of the unincubated egg. An appropriate constant by which to multiply the average result of each method was found by comparing each result with the actual surface as measured with adhesive plaster. By comparing the average errors in the measurement of the surface by each formula, it was pos-

TABLE I.

Measured and Calculated Egg Shell Surfaces Compared.

	Average of eggs (Nos. 1 to 27) ± standard error.*
Measured surface, *sq. cm.*..	75.00 ±0.71
Weight, *gm.*..	56.94 ±0.82
$W^{\frac{2}{3}}$..	14.79 ±0.14
$K = \dfrac{\text{measured surface}}{W^{\frac{2}{3}}}$	5.07 ±0.10
Average deviation of individual, calculated surface from observed surface, *sq. cm.*...	±1.1

* Standard error $= \dfrac{\text{Standard deviation}}{\sqrt{\text{No. of observations}}}.$

sible to estimate approximately which method was the most accurate. The third method for which the equation is $S = 5.07\,W^{\frac{2}{3}}$ gave the most satisfactory results. The average error was ± 1.1 sq. cm. as compared to ± 1.9 and ± 1.6 sq. cm. for the first and second methods respectively. For this reason and because the method dispensed with all measurements except the weight it was selected. (Table I.) It may, of course, be conveniently represented graphically, so that, knowing the weight, the value for the surface can be read off immediately.

Using this formula to obtain the surface area a very slight correlation was found between surface and the amount of weight lost during incubation when other factors such as temperature and humidity

were kept constant. It was then surmised that perhaps the thickness of the shell might be a factor. The thickness of the shell was esti-mated by finding the weight per sq. cm. $\left(\dfrac{\text{Weight of shell}}{S}\right)$. This approximation for the thickness was compared with the average daily weight lost per sq. cm. It was found that the two varied inversely and that the correlation in this case was more definite than in the case of the egg surface. These estimations, however, are necessarily crude since the thickness of the shell is not uniform. An egg with a heavy shell which has very small rarefied areas may lose more weight than one having a lighter shell of uniform thickness. This was shown to be a fact by making very small cracks in the shell, not sufficient to inter-rupt development. The result was that eggs so treated lost 50 to 100 per cent more weight per day than the average.

The conclusion was reached that, although the area and thickness of the shell were among the determining factors in the loss of weight during incubation, the thickness, which seemed the more important of the two, could not be measured with any degree of exactitude, and that, at least for present purposes, if care were taken to choose eggs of approximately uniform size and shell thickness, both functions could be neglected.

The Weight of the Shell during Incubation.

There seems at last to be general agreement about the fact that the shell, the most important element of which is calcium carbonate, loses weight during incubation. The discussion on this point which origi-nated when William Prout (4) first affirmed in 1822 the passage of earthy carbonates into the embryo from the shell, seems to have been largely the result of disregarding the great variability of eggs in almost all their characters. Whatever method is used necessarily involves estimations of the total shell weight or the amount of calcium in the shell or egg contents before and after incubation. As the same eggs cannot, of course, be used for both determinations, and as egg shells before incubation vary at least as much as between 4.5 gm. and 7.7 gm. in weight, or between 7.7 per cent and 11.9 per cent of the total egg weight, it will not be evident whether there is a loss of such a small fraction as 0.3 gm. of shell substance during incubation unless a care-

ful statistical study is made. None of the previous investigators have chosen to do this.

In 1908 Tangl (5) introduced an improvement when he limited himself to a comparison of eggs from the same hen on a standard mineral diet. He believed that his figures showed a loss of 0.3 to 0.4 gm. of shell weight during incubation. His general conclusions have been confirmed by Carpiaux (6) and again more recently by Plimmer and Lowndes (7).

Tangl weighed and analyzed eight dried egg shells before incubation and fifteen after incubation. From his data it may be estimated that the shell weight was 9.64 ± 0.09 per cent[2] of the total egg weight before

TABLE II.

Weight of Shell in Terms of Total Egg Weight before and after Incubation.

Incubation age.	No. of eggs weighed.	Average weight of eggs before incubation.	Average weight of shell.	Average percentage weight of shell.	Average surface area. $S = 5.07 W^{\frac{2}{3}}$	Average weight of shell per sq. cm.	Shell weight in an egg of average weight (57.8 gm.).	Average loss of shell weight.
days		*gm.*	*gm.*		*sq. cm.*	*gm.*	*gm.*	*gm.*
0	35	58.81	5.767	9.81 ±0.11	76.6	0.0753	5.670	
17	25	59.09	5.590	9.46 ±0.14	76.9	0.0727	5.468	0.202
18	16	59.28	5.609	9.46 ±0.09	77.1	0.0728	5.468	0.202
19	22	59.62	5.580	9.36 ±0.12	77.4	0.0721	5.410	0.260

incubation, whereas after incubation it was 9.08 ± 0.10 per cent, the difference between these two averages being 0.56 ± 0.13 per cent. Tangl used a different variety of fowl (Plymouth Rock) from ours in his experiments, and took all his eggs from three hens. We, on the other hand, used eggs from White Leghorn hens and desired average figures representative of the whole flock.

For our purposes it is necessary to know only approximately to what degree, and at what rate the solids of the egg are augmented by the addition of shell constituents. To learn these facts series of egg shells were weighed before and after 17, 18, and 19 days of incubation, respectively (Table II). The weights were obtained after drying the

[2] Unless otherwise stated the designation ± a number refers to ± the probable error; *i.e.*, $0.6745 \dfrac{\text{standard deviation}}{\sqrt{\text{No. of observations}}}$.

shells in an oven.   Before incubation the shells were 9.81 ± 0.11 per cent of the total egg weight; whereas after 17, 18, and 19 days of incubation they were 9.46 ± 0.14, 9.46 ± 0.09, 9.36 ± 0.12 per cent, respectively, of the initial egg weight, the difference being consequently 0.35, 0.35, and 0.45 per cent.   This might be compared to the difference found by Tangl after incubation, namely, 0.56 per cent. The greater loss of weight in his experiments is probably due to the fact that his incubated eggs were examined later, after 20 days of incubation.   Tangl obtained greater differences in a later but smaller set of weighings (8).
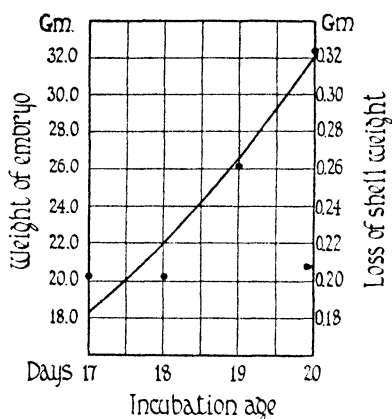
FIG. 1. A comparison of the weight of the embryo (heavy line) as determined by the formula $W_e = K\ t^{3.6}$ and the average loss of shell weight (black circles (●)) as calculated from a series of weighings (Table II) when expressed as functions of the incubation age.

The differences are not great as judged by the criteria of statistical theory, and inasmuch as our results are comparable to those of Tangl, who had the advantage of working with eggs from the same hen, the data may be taken to afford a basis for an approximation which would probably allow for greater accuracy in computing the changes in concentration of total solids during incubation.   If there is a transfer of substance from the shell into the interior of the egg it must be known at what rate this occurs.   Tangl showed a loss of 0.56 per cent in shell weight after 20 days of incubation.   In an egg weighing 57.8 gm. this is equivalent to 0.324 gm. of shell substance.   Using this figure

together with our own results, and plotting the loss of shell weight against the incubation age, it may be seen (Fig. 1) that the loss of weight increases with the wet weight of the embryo, the latter being represented in the figure by the curve. This fitting may be coincidental, but without the collection of a much greater number of observations it may serve as a basis for a simple approximation which would lead to a greater degree of accuracy than would be obtained by neglecting time as a function in the loss of shell weight or neglecting the latter altogether. Carpiaux, as well as Plimmer and Lowndes, found approximately 0.04 gm. of CaO in the egg contents before incubation and 0.20 gm. after incubation. Now, as the work of Tangl and Carpiaux demonstrated no change in the constitution of the shell during incubation, and as the average of eleven analyses by Plimmer and Lowndes gave the CaO as approximately 50 per cent of the total shell weight, it would seem that the loss of shell weight during incubation was about 0.32 gm., a figure which almost exactly corresponds to our own. Plimmer and Lowndes in one series determined the lime in the egg contents on consecutive days. Their average results, when multiplied by 2 to show the loss of shell weight, follow the trend of our graph, but are all somewhat lower. Since their chicks hatched later than ours, it might well have been that the embryos were larger in our series and thus more lime had been absorbed.

From the graph it is seen that for every 1.0 gm. of embryo there is a loss of 0.01 gm. of shell substance, that is to say a gain of 0.01 gm. of total solids in the egg contents.

To obtain the weight of the shell before incubation, the following formula may be used:

$$L_0 = L_t + 0.01 \, W_e \qquad (1)$$

where L_0 = the weight of shell before incubation, L_t = the weight of the shell after t days of incubation, and W_e = the wet weight of the embryo.

It will be shown in a later paper that the weight of the chick embryo between the 5th and the 19th days of incubation may be expressed by the equation

$$W_e = \frac{t^{3.6}}{1.496}$$

where W_e = the weight in mg. of the embryo, and t = the incubation age in days.   For L, the weight of solids derived from the shell and added to the egg contents, the following equation may be used.

$$L = \frac{t^{3.6}}{149.6} \tag{2}$$

These figures would be subject to a correction if it were known that some of the carbonates from the shell were transformed into carbon dioxide gas and as such passed out into the atmosphere.   The metabolic determinations would likewise be affected.   In the absence of the necessary data calculations have been made without correction as if the probable retention of CO_2, due to the association of calcium carbonate, upon its dissolution from the shell, with carbonic acid to form bicarbonate,   was compensated   by the delivery   of an equal amount of CO_2 when the calcium is reprecipitated to form bone in the embryo.

The   Concentration of   Water,   Total   Solids,   and   Fat   during Incubation.

The first experiment on the loss of weight during incubation was done while using a Freas incubator which one was required to open for other purposes several times a day.   Thirty-three eggs were weighed every day throughout the incubation period as a preliminary test. There was found to be an equal distribution of fertile, unfertile, and degenerated eggs and these were all averaged together.   The mean weight of all the eggs for each day was plotted against the time (Fig. 2). The diagram shows that the daily weight decrement is constant. The temperature was usually 38°C. in the incubator, but as the doors were opened periodically it dropped below this value at times.   The presence of an electric fan which circulated the air over the eggs hastened evaporation.   Once a day each egg in a large weighing bottle was removed into laboratory conditions for weighing.   At this time it was rolled.   The humidity in the incubator was not measured.

In subsequent experiments information was sought on the effect of what we considered to be the two most important variable factors affecting incubation, namely (1) temperature and (2) humidity. Since poultrymen have learned by experience that it is important to

roll eggs regularly, the surface of each egg was divided into three numbered sections by equidistant longitudinal axial lines. The eggs were placed on small rubber rings so that there should be no free motion and laid in the drawers so that their sides touched. When rolled, each egg was turned two-thirds of the way round, so that the segment number next but one pointed upwards. It was found that among the eggs rolled twice a day there was a higher percentage of live embryos than among those rolled once a day or once every other
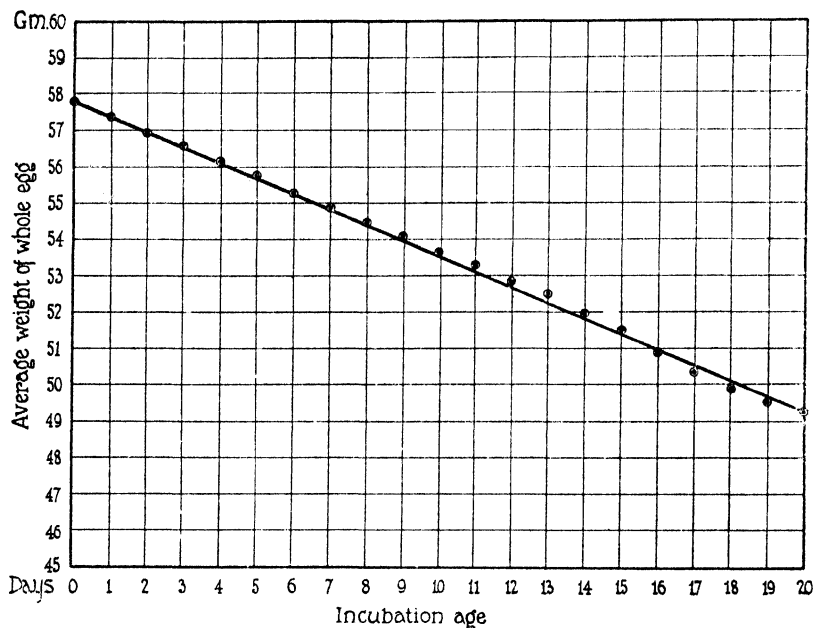
FIG. 2. The average weight of thirty-three eggs, fertile and unfertile, during the entire period of incubation.

day. But since neither the weight nor the chemical constitution of the eggs was found to vary in any regular fashion, this factor was not investigated further.

With the temperature constant three series of eggs were incubated at different concentrations of water vapor and periodically weighed. The humidity was determined by the use of dry and wet bulb thermometers. Eggs were opened on the 17th, 18th, and 19th days and the embryos therein as well as the rest of the egg contents were

analyzed for solids and fats. For the most part there were no differ-
ences in the weight lost by fertile and unfertile eggs (Table III, Fig. 3),
between the 4th and 14th days of incubation the points in all cases
approximated a straight line. It is therefore possible to compare the
average loss of weight per day of eggs at different humidities. The
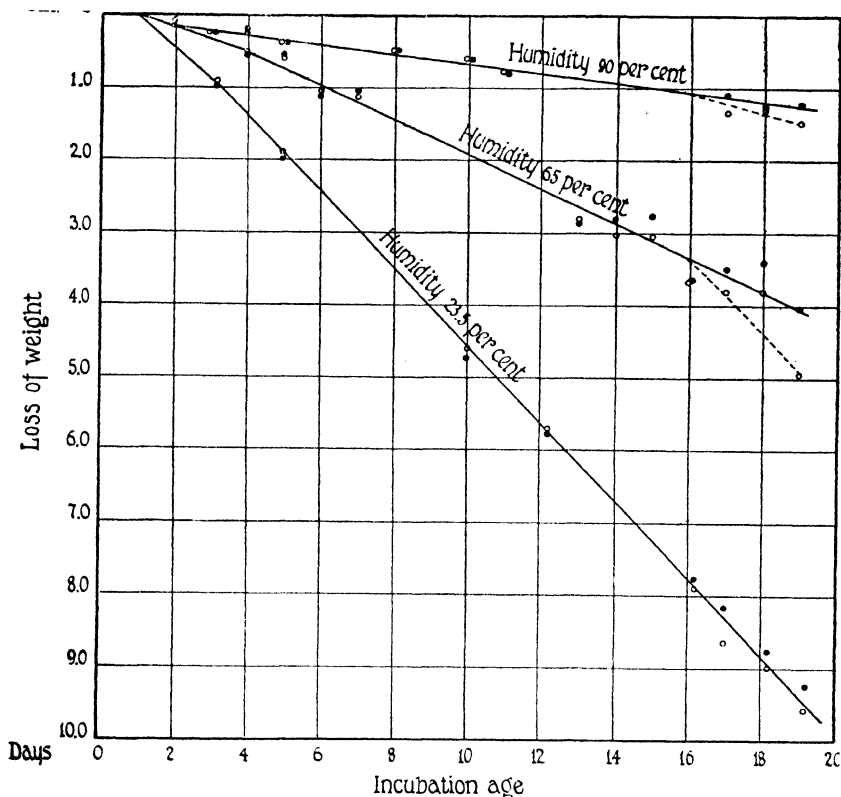
FIG. 3. The average weight of fertile (white circles (○)) and unfertile (black
circles (●)) eggs, incubated at humidities of 90, 65, and 23.5 per cent respectively,
equated against time (Table III).

deviations from the straight line in the middle period it was thought
were due to minor fluctuations in the humidity, a factor which it was
never possible to keep quite constant. The humidity of the atmos-
phere, apparently, determines the amount of weight lost during incu-
bation (Fig. 4). This leads to the inference that the loss of weight

TABLE III.

Loss of Weight of Incubated Eggs under Different Conditions of Humidity.

Conditions.	Fertile or unfertile.	No. of eggs.	Average weight of egg.	Average weight of shell.	Average loss of weight per day.
			gm.	*gm.*	*gm.*
Temperature 38.2°. Humidity 90 per cent.	F.	23	57.82	5.409	0.066
	Unf.	29	60.18		
Temperature 38.2°. Humidity 65 per cent.	F.	12	60.04	5.596	0.242
	Unf.	19	57.70		
Temperature 38.2°. Humidity 23.5 per cent.	F.	8	57.14	5.375	0.533
	Unf.	8	58.25		

Loss of weight.

Temperature 38.2°. Humidity 90 per cent. — Incubation days.

	2	3	4	5	8	10	11	17	18	19
	gm.	*gm.*	*gm.*	*gm.*	*gm.*	*gm.*	*gm.*	*gm.*	*gm.*	*gm.*
F.	0.11	0.25	0.27	0.39	0.48	0.62	0.79	1.36	1.28	1.49
Unf.	0.14	0.25	0.26	0.40	0.49	0.61	0.81	1.11	1.26	1.23

Temperature 38.2°. Humidity 65 per cent. — Incubation days.

	4	5	6	7	13	14	15	16	17	18	19
F.	0.49	0.59	1.10	1.13	2.82	3.04	3.05	3.69	3.83	3.82	4.96
Unf.	0.52	0.56	1.14	1.14	2.88	2.81	2.79	3.68	3.51	3.41	4.06

Temperature 38.2°. Humidity 23.5 per cent. — Incubation days.

	3.2	5	10	12.2	16.2	17	18.2	19.2
F.	0.96	1.91	4.61	5.71	7.93	8.66	9.01	
Unf.	1.00	2.00	4.75	5.79	7.78	8.19	8.78	9.26

is due to the evaporation of water.   For instance, when there is 100 per cent humidity the egg loses no weight.   The equation for this line is:

$$H_a = 7.5 \ (100 - h) \tag{3}$$
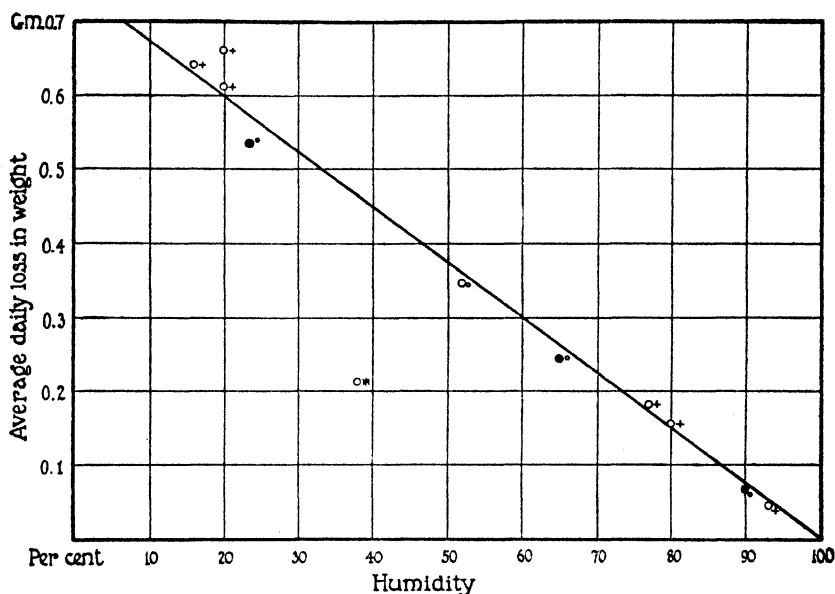
FIG. 4. The average daily loss of weight of incubated eggs as a function of the humidity.   The black circles (●) represent averages of a relatively large series of eggs over the entire period of incubation; the white circles (○) are averages from a small series of eggs measured over a 2 day period.   Points marked with the further notations + and ° and * refer to eggs kept at temperatures above 38.8° (+), just below 38.8° (°), and at laboratory temperature (*) respectively (Table IV).

where H_a = the average daily loss of weight (mg.) and h = humidity in per cent, and thus

$$H = 7.5 \ (100 - h)t \tag{4}$$

where H = weight in mg. of the water lost by evaporation in t days of incubation.

As a confirmation of the initial findings a few shorter experiments were performed (Table IV).   Each white circle (○) (Fig. 4) on the

chart represents the average loss of weight of about a dozen eggs measured over a 2 day period.   The temperature was not the same in all these experiments.   The points marked with a cross ($+$) represent experiments in which the average temperature was above, those with a circle ($°$) experiments in which the temperature was below 38.8°C. It appears that increasing the temperature augments the amount of weight lost by evaporation.   The point far below the line in this figure was the result obtained when eggs were left at a laboratory temperature.

TABLE IV.

Average Loss of Weight per Day of Eggs Incubated at Various Humidities.

No. of eggs weighed.	Average weight of eggs.	Temperature.	Humidity.	Average weight lost per day.
	gm.	*°C.*	*per cent*	*gm.*
4	58.0	39.6	93	0.044
52	57.7	38.2	90	0.066
12	54 3	39.2	80	0.155
12	58.0	39.2	77	0.180
31	60.6	38.2	65	0.242
10	58.8	38.5	52	0.345
10	59.0	21.7	38	0.220
16	57.1	38.2	23.5	0.533
12	59.8	39.2	20	0.610
4	61.2	39.2	20	0.660
16	60.8	40.6	16	0.640

There are factors of less importance.   When eggs are isolated so that neither the sides of the drawer nor other eggs impinge upon them they lose more weight, and this occurs also when an electric fan blows air over their surfaces.   Both arrangements serve to decrease the humidity in the immediate vicinity of the eggs and thus to favor evaporation.

In other experiments we have made certain that for our purposes no account need be taken of the slight differences in average daily weight loss which are occasionally found during the first 2 or 3 days of incubation.   When the eggs are first put into the incubator they are cold and their water content is variable.   Due to such factors the loss of weight which commences immediately may be less regular and different from the constant rate attained later.

The divergence on the part of the fertile eggs from the straight line during the last 3 or 4 days of incubation (Fig. 3) may, however, be of some significance to this study. Its explanation may furnish a clue to metabolic changes of importance. Any hypothesis descriptive of the phenomenon must take into account the following experimental facts: (1) Whereas the unfertile eggs (●) continue losing weight at a constant rate, the daily decrement for the fertile (○) eggs becomes greater. It cannot be due, as was first thought, to an unusually long exposure (about 1 hour) to a humidity of 23 per cent while some of the eggs were being weighed and opened, because the unfertile eggs (●) which were used as controls, were subjected to the same conditions. (2) The extra loss of weight is slight, relative to the average daily decrement in eggs incubated at 23.5 per cent humidity, but in those kept at humidities of 65 and 90 per cent the increase in the daily loss during these last days of incubation is approximately equal to the previous average daily loss.

Three possible interpretations of the observed phenomenon present themselves: (1) that the expired carbon dioxide is greater by weight than the oxygen inhaled during this period, (2) that some other gaseous product is eliminated, and (3) that there is an increased evaporation of water. The first two possibilities apparently do not fit the facts. As embryos, of the same age, developed at humidities of 65 and 90 per cent, respectively, were approximately of equal weight regardless of the humidity, the increased decrement, which was *different* for each humidity, could not have been due to the loss of some metabolic product, such as carbon dioxide because the latter being a function of some embryonic dimension would have been of the *same* magnitude under both conditions. Furthermore, if the loss of weight is due to the carbon dioxide output it must be the result of protein catabolism, since glucose is present in such small quantities that it may be neglected and when fat is burned the oxygen absorbed is greater by weight than the carbon dioxide lost. The urinary function in the chick has been said to start during the 2nd week and there is usually some fecal material in the amniotic cavity by the end of incubation. Even though no nitrogen is lost, there may be and probably is some protein catabolism, the products being eliminated in the usual forms, such as urea, urates, creatinine, and amino acids into the amniotic

fluid. Whether some of these nitrogen catabolites are reabsorbed is unknown. It seems that but little protein is burned during incubation, because in the metabolic experiments of Bohr and Hasselbalch (9) the respiratory quotient, during the latter half of incubation at any rate, was found to be about 0.710. Tangl's experiments (8) also point to the fact that practically all the energy involved is at the expense of fat.

In considering the effect of the gas exchange upon the weight of the egg, reference may be made to the estimations of Loewy which indicate that for every 100 cc. of CO_2 eliminated as a result of protein metabolism there is a loss of 0.0181 gm. of weight; whereas for every 100 cc. of CO_2 from fat there is a gain of 0.0056 gm. of weight (Table V). Even if one assumes that protein contributes as much as 20

TABLE V.

The Respiratory Exchange and the Change in Weight When Protein and Fat Are Oxidized (after Lusk[3]).

1 gm. of solid.	Respiratory gases.				Difference in weight per gm. of solid burned.	Difference in weight per 100 cc. CO_2 expired.
	Oxygen.		Carbon dioxide.			
	cc.	gm.	cc.	gm.	gm.	gm.
Protein.	966.3	1.380	773.9	1.520	−0.14	−0.0181
Fat.	2,019.3	2.885	1,427.3	2.805	+0.08	+0.0056

per cent of the CO_2 expired daily during the latter part of the incubation period, the loss from this source ($0.0181 \times 0.20 = 36.2 \times 10^{-4}$) would be more than neutralized by the gain in weight from fat metabolism ($0.0056 \times 0.80 = 44.8 \times 10^{-4}$). Much careful experimentation has been done by Hasselbalch (10) and others to discover whether ammonia or some other N-containing gas is eliminated by fertile eggs. It was generally believed that some small variable fraction of expired air was in this form, until the work of Krogh (11) and Tangl (8) which seemed to show that there was no loss of nitrogen during incubation. Our experiments are not intended to settle this point. The literature on the subject has led us to believe that its solution is of no particular

[3] Lusk, G., The elements of the science of nutrition, Philadelphia and London, 3rd edition, 1921, 62.

moment to our problem, since the amount of weight, if any, lost in such a form would appear to be practically negligible. Therefore, by a process of elimination we are led to the conclusion that the increased loss of weight during the latter part of incubation is due to an excessive elimination of water.

It appears consequently that changes taking place in fertile eggs result in a modification of the factors conditioning evaporation. The temperature of the living organism is higher than that of the surrounding atmosphere, and the amount of heat manufactured is roughly proportional to the mass of active tissue. Therefore, when the embryo has attained significant size, the heat radiating from its surfaces modifies temperature conditions in the whole egg. We have observed under standard conditions that a thermometer placed against the shell of a fertile egg gives a reading, the height of which above the surrounding temperature varies in general with the weight of the embryo. The change is not noticeable until near the end of incubation. As vapor tension is a function of temperature, it would seem on theoretical grounds that in consequence of the amount of heat produced by embryos of advanced age there should be an acceleration of evaporation, other factors being equal, during the last few days of incubation. Possibly the circulation of blood in the respiratory membrane which at this period covers the inner surface of the shell affords a more effective evaporating mechanism than the undifferentiated albumin. Finally, as will be shown later, the concentration of water in the egg rises during these last few days of incubation due to catabolic processes within the embryo so that for this reason the vapor tension and, consequently, the evaporation of water may be increasing. These suggestions are offered as explanations of the increased increment loss of weight during the last days of incubation. In the eggs incubated at very low humidities (23 per cent), however, there seem to be factors within the egg which limit evaporation.

In view of such considerations regarding the excessive evaporation of water at humidities between 65 and 90 per cent greater accuracy is obtained on the 17th, 18th, and 19th days of incubation by substituting for equation (4) the following

$$H = 7.5 \ (100 - h)t + 7.5 \ (100 - h) \ (t - 16) \tag{5}$$

where H = water lost by evaporation (mg.), h = humidity (per cent), and t = incubation age (days).

The equations which are now available express approximately the daily loss of weight of the whole egg (and thus the evaporation of water) as a function of the humidity, and the loss of weight of the shell (and thus the gain in weight of total solids within the egg) as a function of time. At any time during incubation by the use of one or more of these equations the original weight of the contents of any egg may be estimated, and thus an approximate value for the quantity of solid material *before* incubation may be obtained. By comparing the latter with the amount of solid material actually found *after* incubation it should be possible, despite the great variability of the eggs, to calculate within limits how much solid material is burned during incubation.

TABLE VI.

Analysis of Unincubated Eggs.

	No. of eggs analyzed.	Per cent of egg contents.	Per cent of total solids.	Gm. per 100 gm. H₂O.
Total solids................................	37	24.7 ±0.1	100	32.8
Fat..	11	11.1	45.0 ±0.8	14.7
Nitrogen...................................	9	1.9	7.6 ±0.1	2.5
Protein (6.25 N)............................		11.7	47.5	15.5

The water content of the egg was obtained by subtracting from the weight of the whole egg the dried weight of the contents and the dried shell. Before drying the shell the albuminous material adhering to the inner surface of the shell membrane was washed off with distilled water and included with the rest of the egg contents. The egg substance was dried, (1) exposed to the air in an oven at 102°C.; (2) in a vacuated desiccator over H_2SO_4 at 60°C.; and (3) in a vacuated desiccator over phosphorous pentoxide at 102°C. The results obtained by the three methods were comparable, and as the first was the simplest it was thenceforth used exclusively.

The average concentration of water in thirty-seven eggs before incubation as they were received in the laboratory was 75.3 ± 0.1 per cent. The concentration of solids was therefore 24.7 ± 0.1 per cent (Table VI).

Let

W = weight of whole egg before incubation.
L_0 = weight of shell before incubation.
L_t = weight of shell after t days of incubation.
W_e = weight of embryo.
S_0 = weight of solids before incubation.
S_t = weight of solids after t days of incubation.
S = weight of solids burned.

Now since

$$L_0 = L_t + 0.01 \ W_e \qquad (1)$$

and the solids represent 24.7 per cent of the egg contents, then

$$S_0 = 0.247 \ (W - L_t - 0.01 \ W_e)$$

and

$$S = 0.247 \ (W - L_t - 0.01 \ W_e) - S_t$$

The terms on the right side of the equation are all measurable.

Two sets of fertile eggs incubated at 90 and 65 per cent of humidity respectively were analyzed after incubation (Table VII). They are peculiarly irregular, but the averages seem to show a certain uniformity. As will be seen later there are reasons to believe that these are sufficiently accurate for our purposes.

When the humidity of the incubator air is changed, there is an accompanying change in the concentration of substances composing the yolk and albumin due to the evaporation of water of the eggs. Within rather wide limits such modifications have, however, no measurable effect upon the development and chemical differentiation of the embryo. The passage of substances into the embryo is not conditioned to any great extent by such external factors. As with many glands and other organs in the body, there appears to be evidence of selective absorption, so called, which to a large extent is independent of the concentration or total mass of the substance to be absorbed. The devices used to symbolize membrane equilibria in non-living colloidal systems are therefore not descriptive of the phenomena under observation; and one must resort in the present state of ignorance to certain specifically biological concepts. Haldane (12) believes, it seems, that the latter are axioms, characteristic of

TABLE VII.

Solids Oxidized during Incubation.

Humidity (per cent)	Incubation age (days)	Egg No.	W (gm.)	L_t (gm.)	W_e (gm.)	W_0 (gm.)	$S_0 + L$ (gm.)	S_t (gm.)	S (gm.)	$\frac{S_t}{H_t}$ (gm.)
						Average ± standard error.*				
90	17	4,7,8,9,19, 22,26,27.	58.93 ± 0.84	5.27 ± 0.23	20.35 ± 0.39	53.46 ± 0.67	13.41 ± 0.17	12.24 ± 0.21	1.16 ± 0.06	30.6 ± 0.2
	18	41, 45.	51.98 ± 1.84	5.00 ± 0.20	22.15 ± 1.35	46.75 ± 1.67	11.77 ± 0.40	10.58 ± 0.76	1.19 ± 0.35	29.9 ± 1.3
	19	32,46,48, 54,53.	59.08 ± 1.88	5.62 ± 0.16	29.64 ± 0.67	53.15 ± 1.73	13.42 ± 0.43	11.63 ± 0.50	1.79 ± 0.22	28.8 ± 0.72
65	16	77†	58.49	5.22	14.45	53.12	13.26	12.46	0.80	34.1
	17	84,86,73.	60.77 ± 1.11	6.20 ± 0.23	17.48 ± 0.32	54.39 ± 0.68	13.60 ± 0.17	12.61 ± 0.10	0.99 ± 0.14	33.4 ± 0.51
	18	56,58.	57.74 ± 1.79	5.42 ± 0.10	20.78 ± 1.65	52.10 ± 1.68	13.07 ± 0.42	11.86 ± 0.78	1.20 ± 0.35	32.3 ± 1.5
	19	60,78,79, 90,76.	62.07 ± 2.12	5.75 ± 0.22	25.78 ± 1.12	56.06 ± 1.95	14.09 ± 0.54	12.55 ± 0.48	1.53 ± 0.23	32.5 ± 0.85

* Standard error $= \dfrac{\text{standard deviation}}{\sqrt{\text{No. of observations}}}$.

† Individual determination.

W = weight of the whole egg before incubation; L_t = weight of shell.

W_e = weight of embryo; $W_0 = W - L_t - 0.01$; W_s = weight of egg contents before incubation.

$S_0 = 0.247$; W_0 = weight of solids before incubation.

$L = 0.01$; W_s = weight of solids added to egg contents; $S_0 + L$ = solid content on the assumption that no solids are oxidized;

S_t = solid content as found; S = solid burned.

$\dfrac{S_t}{H_t}$ = solids in whole egg (gm. per 100 gm. of H_2O).

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and peculiar to living organisms and not to be resolved into chemical and physical terms. In pursuit of this general idea and in order to gain information of the phenomena underlying the movement of elements in and out of the embryo (absorption and elimination) it is desirable to know in approximate terms the changes in the concentration of the more important constituents of the whole egg during incubation.

It was necessary to know not only what substances are burned but also at what rates. In view of the literature on the subject the solids were analyzed for fat. The results may be seen in Table VIII. The fat content was determined by alcohol-ether followed by pure ether extraction in a Soxhlet apparatus. Before extraction and once during the latter part of the extraction process the dried substance was thoroughly ground in a mortar. The extraction process lasted about 20 hours. After extraction the flasks were dried to constant weight over paraffin in a vacuated desiccator. The term "fat" is henceforth used as synonymous with the extract thus obtained.

Since individual eggs show considerable variation our results are not very regular. The sensible maximum deviations in the content of total solid and fat before incubation (Table VI) were 1.6 per cent and 4.0 per cent, respectively; this amounts, then, to a possible error of $\pm$ 0.8 gm. in our calculations for S_0 (solid content before incubation (Table VII)) and approximately $\pm$ 1.3 gm. for F_0 (fat content before incubation (Table VIII)), since the latter estimation involves both errors. The maximum deviations from the mean in our values for S, the amount of solid burned during incubation, were well within these figures (Table VII), and therefore we were not led to seek further for an explanation of the apparently irregular variations. A comparison between the total amount of solid substance (28.362 gm.) and of fat (28.059 gm.) lost during incubation by twenty-two eggs shows a close correspondence (Table VIII). Approximately 98 per cent of the oxidized solid substance seems to be fat. This is corroborative of previous work, but may not be more accurate than $\pm$ 10 per cent. Tangl and von Mituch (8) found by the analysis of the contents of six fertile eggs after the incubation period that practically all the loss of solids could be accounted for by the oxidation of fat. Bohr and Hasselbalch (9) came to the same conclusion when they found that

TABLE VIII.

Fat Oxidized during Incubation.

Humid-ity.	Incu-bation age.	Egg No.	W_e	W_0	S_0	F_0	F_t	F
					Average ± standard error.*			
per cent	days		gm.	gm.	gm.	gm.	gm.	gm.
90	17	4,8,9,19,22,26,27.	20.24 ± 0.43	53.57 ± 0.77	13.23 ± 0.19	5.95 ± 0.09	4.92 ± 0.12	1.04 ± 0.12
	18	41†	20.32	49.09	12.13	5.45	4.14	1.31
	19	32,46,48,54,53.	29.65 ± 0.67	53.15 ± 1.74	13.13 ± 0.41	5.90 ± 0.19	4.28 ± 0.36	1.63 ± 0.26
65	16	77†	14.46	53.12	13.12	5.90	5.13	0.77
	17	86,73.	17.65 ± 0.44	53.63 ± 0.41	13.24 ± 0.10	5.96 ± 0.05	4.95 ± 0.11	1.01 ± 0.06
	18	56,58	20.78 ± 1.65	52.10 ± 1.68	12.87 ± 0.41	5.79 ± 0.18	4.57 ± 0.55	1.22 ± 0.37
	19	60,78,79,76.	25.10 ± 1.17	54.60 ± 1.9	13.49 ± 0.47	6.07 ± 0.20	4.54 ± 0.21	1.53 ± 0.17

Total F . . . . = 28,059 gm.

" S (Table VII) from same eggs (*i.e.* omitting Nos. 7, 45, 84, and 90) . . . . = 28,362 "

* Standard error = $\dfrac{\text{standard deviation}}{\sqrt{\text{No. of observations}}}$.

† Individual determination.

W_e = weight of embryo; W_0 weight of egg contents before incubation (from Table VII).

S_0 = 0.247; W_0 = weight of solid before incubation.

F_0 = 0.45; S_0 = weight of fat before incubation; F_t = weight of fat after t days of incubation.

F = fat burned.

the respiratory quotient for chicken embryos during the greater part of incubation was approximately 0.710. It must be kept in mind that the available figures show irregular variations, that the respiratory quotients for egg fats and proteins are not accurately known and that other transformations (carbohydrate formation from protein or fat) may take place within the embryo to vitiate conclusions from respiratory data. Nevertheless in view of the literature and our own confirmatory data it seems that *for our calculations* it is a justifiable assumption that fat only is oxidized during incubation.

There are certain reasons, as Tomita (13) and more recently Needham (14) have pointed out, for supposing that some glucose is metabolized before the 10th day of incubation. In the first place Bohr and Hasselbalch found a respiratory quotient of 0.89 as an average for the first 5 days of incubation; secondly the trace of glucose, which is known to be present at the start of incubation, is no longer to be found in the egg contents outside of the embryo after the 1st week of incubation (15, 16); and finally the curve for the concentration of lactic acid in the whole egg which was constructed by Tomita as a result of a large number of determinations, and is an index according to the author of glucose metabolism, rises at the start of incubation, reaches its maximum on about the 4th day, and then declines.

It is uncertain when the end-products of nitrogen metabolism first appear in the allantoic sac; but if experiments were to prove that there was a procession of glucose, protein, and fat as sources of energy the findings might be correlated to the change with age in the value of the respiratory quotient as portrayed in Needham's graphical representations of Bohr and Hasselbalch's figures. Later it may be possible to determine the truth of this hypothesis and to estimate how much carbohydrate and protein metabolism occurs. The embryo's mass is so insignificant in the beginning of incubation that during the first $9\frac{1}{2}$ days (one-half of the period under observation) only one-seventeenth of the amount of solid substance eventually to be burned is lost. There would probably be an error of no more than 5 per cent if the catabolism of the first half of incubation were calculated on the basis of fat, instead of carbohydrate and glucose.

Assuming then that only fat is oxidized, determinations were made of the rate of fat catabolism at each incubation age. There were

before us the tabulations of total oxidized solid (Table VII) for the 16th to the 19th day inclusive, but these were not sufficient to extrapolate earlier values.   There were also at hand the careful researches of Bohr and Hasselbalch (9, 17) on the metabolism of chicken embryos; but in their experiments the breed of the hen laying the egg was not recorded, the conditions of incubation were not fixed, the chicken weights for some ages were quite different from our own, the weights of the embryos actually used in the metabolic tests were not determined, and finally inspection revealed unusually large variations in the results.   It was therefore considered necessary to determine for ourselves the carbon dioxide expiratory rate throughout incubation.

Carbon Dioxide Production.

The apparatus used was similar to the conventional pattern (Fig. 5).   Air was drawn by suction at a constant rate through soda-lime and water vapor into a glass bottle containing the egg, and then successively through sulfuric acid (tube G), soda-lime (tube H), and sulfuric acid (tube J).   The difference between the combined weights of tube H and tube J, before and after a certain interval of time, is the weight of carbon dioxide eliminated by the egg in that time.

The following experiments were made and repeated in order to test the efficiency of the apparatus:

(1) Barium hydroxide in tubes through which CO_2-free air had been passed were placed in the circuit after vessel C and tube J.   Absence of turbidity, or a precipitate, indicated that the CO_2 was being properly absorbed.

(2) A second sulfuric acid tube was put into the circuit after tube G.   No change in its weight after a long period of circulation indicated that tube G was absorbing all the water.

(3) Air was circulated for several hours through the empty apparatus.   There was no change in the combined weight of tubes H and J.

The rate of flow of air which was nearly saturated with water in all experiments was constant at 3 liters per hour, so that in the larger embryos only did the surrounding atmosphere approach a concentration of CO_2 as great as 0.5 per cent.   In some of Hasselbalch's experiments the concentration rose above 0.5 per cent without any noticeable change in the metabolic rate, according to the authors, so that we were led to consider this a factor of little significance.   It was found

that within wide limits a change in the humidity during the short time that the test lasted did not affect the rate of CO_2 elimination.

Bohr and Hasselbalch laid considerable emphasis upon the elimination of CO_2 by the shell. In these experiments it was assumed that after carbonic acid equilibrium had been fixed between the egg and the surrounding atmosphere there would be no measurable interchange in either direction, and that this condition could be achieved by allowing for a preliminary period of circulation before commencing the experiment. No test was commenced until air had flowed through the apparatus at the same constant rate for at least half an hour in
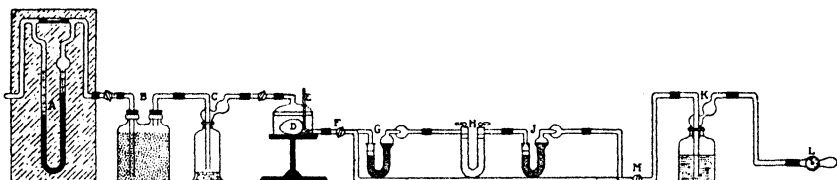
FIG. 5. Diagram of apparatus set up on a table in a constant temperature room to measure the carbon dioxide production of chicken embryos.

A = flow-meter; B = bottle of moist soda-lime; C = bottle with a small quantity of barium hydroxide solution at the bottom for the purpose of moistening the air passing over it and of giving evidence, by the formation of a thin superficial film, of the presence of any CO_2 in the air; D = egg resting in a glass bottle with two outlets and a ground glass stopper through which passes E; E = thermometer, the bulb of which lies against the shell; F = two-way stop-cock; G = pumice and sulfuric acid tube; H = moist soda-lime tube; J = pumice and sulfuric acid tube; M = two-way stop-cock; K = trap; L = dial on a needle valve to indicate flow of air into suction tube.

the older embryos and for an hour in the embryos less than 10 days old. In a number of the experiments values were obtained for the CO_2 output of single embryos over short successive periods. The lack of a regular decline or increase in their metabolism showed that a plateau or a relatively constant rate is attained after half an hour.

Mr. J. B. S. Haldane has called my attention to the fact that as the egg white has an alkaline reaction during the early stages and is said later to become acid (18), the change in pH may be due in part to the retention of CO_2 formed in metabolism. The figures for CO_2 elimination, particularly for the younger embryos may accordingly be too

low.   Moreover, as mentioned above, the influence of the carbonates from the shell is at present undetermined.   As they enter into solution there may be a retention of CO_2 and as they become reprecipitated in the bone, there may be an equivalent liberation of the gas.   If these

TABLE IX.

Carbon Dioxide Production of Chicken Embryos.

Experiment No.	Egg weight.	Age.	Embryo.			Temperature.	Duration of test.	CO_2 per 24 hrs.	CO_2 per 24 hrs. per gm.
			Weight.	Log weight.	Sex.				
	gm.	days	mg.			°C.	hrs.	cc.	cc.
1	57.8	6	330	2.52	M.	38.5	5	9.5	28.6
2	59.4	6	526	2.72		38.0	6	15.7	29.8
3	60.5	8	815	2.91		38.8	6	25.2	30.9
4		7	832	2.92		38.7	3	25.1	30.1
5	57.0	8	1,213	3.08	"	38.9	4	31.5	26.1
6	58.3	9	1,542	3.19		38.8	4	46.3	30.0
7	58.9	9	1,726	3.24	"	38.7	6	50.9	29.5
8	59.9	10	2,534	3.40		38.8	5	70.3	27.8
9	59.5	11	3,494	3.54	F.	38.7	5	94.5	27.1
10	60.2	11	3,650	3.56	M.	38.7	1	101.8	27.8
11	58.3	12	3,812	3.58	F.	38.9	1	106.9	28.0
12		12	5,411	3.73	M.	38.8	2	150.3	27.8
13	57.1	13	8,629	3.94	F.	39.0	1½	213.3	24.7
14	57.1	14	10,618	4.02		38.8	1	259.3	24.7
15	58.0	14	11,562	4.06		37.8	1	261.7	22.6
16	59.1	14	12,113	4.08	"		1	230.3	19.0
17	56.4	15	14,862	4.17	"	39.1	1½	322.4	21.7
18	56.6	16	15,105	4.18	"	39.0	1½	320.0	21.2
19	58.4	16	18,229	4.26	M.	39.0	1	363.6	19.9
20	58.7	18	22,314	4.35	F.	38.7	1½	373.3	16.7
21	56.3	17	22,745	4.36	"	38.6	1	387.8	17.0
22	63.6	18	25,850	4.41	"	38.9	5½	357.0	13.8
23		16	15,826	4.20	M.	38.9	1	326.0	20.6
24		17	18,181	4.26	"	38.8	1	348.0	19.1
25		18	23,968	4.38	F.	38.8	1	358.0	16.9
26		19	25,218	4.40	"	38.7	1	368.0	14.6

two processes are not occurring simultaneously there will be errors in the figures for metabolism.   Some of the more evident sources of error such as these will be investigated at a later date, but in the present study they have been neglected.

The results of our experiments have been tabulated (Table IX) and

the CO_2 production per 24 hours, equated against the logarithm of the embryonic weight (Fig. 6). Through the points on the chart a smooth curve may be drawn and then may be read off on the curve the values (Column 2, Table X) which correspond to the logarithms of the weights reported elsewhere as the averages for their respective ages (Column 1, Table X). This indirect method of arriving at the CO_2 production rate in terms of incubation age is more accurate, since
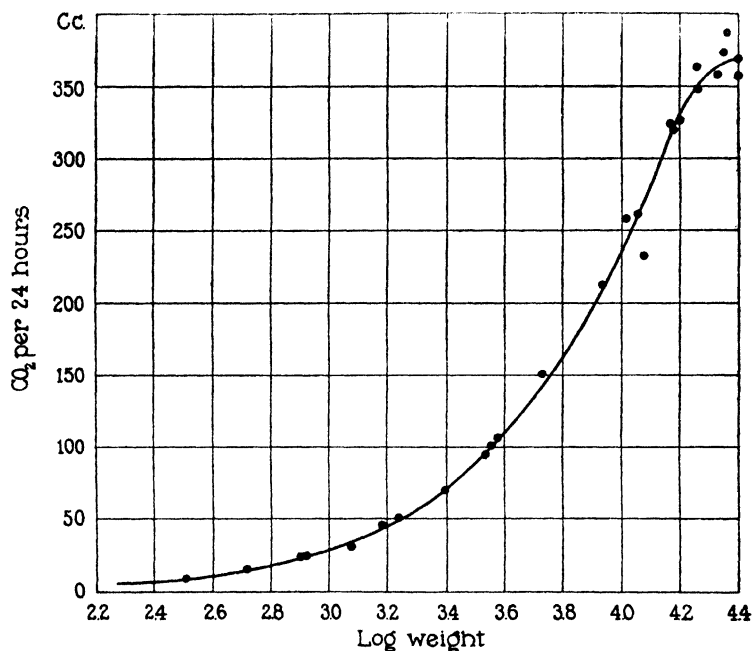
FIG. 6. The CO_2 production per 24 hours equated against the logarithm of the wet weight of the embryo. The graph to describe theoretically the locus of the points was drawn by inspection (Table IX).

the correlation of catabolism with embryonic weight is closer than with age. The average embryonic weight at each age has been fixed with relative certainty by several hundred weighings. It is therefore justifiable to draw the curve expressing the rate of CO_2 production in terms of embryonic age (Fig. 7). By measuring with a planimeter the area under the graph for each day, the integrals may be obtained which give the total CO_2 elimination since the beginning of incubation

In the eggs incubated at 90 per cent humidity the embryos were in general somewhat larger, but it was quite common to find mold colonies adherent to the inner surface of the shell. Due to the fact that the humidity under a hen is said to be about 60 per cent and that our eggs kept under these conditions (65 per cent) gave the highest percentage of live embryos, it was decided to maintain the humidity
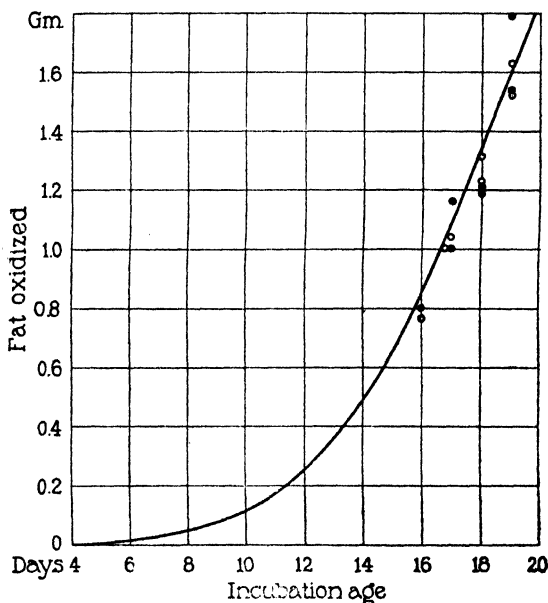
FIG. 8. A curve to show the total amount of solid (or fat) oxidized during incubation when the assumption is made that fat only is burned. The graph may be compared to the black circles (●) which represent total solids, and the white circles (○) which represent fat as actually determined (Tables VII and VIII).

constantly between 65 and 70 per cent (average 67.5 per cent) and the temperature at 38.8 ± 0.4°C.

With these data at hand one can construct a theoretical table, which will give the content of total solids, fat, and nitrogen for each day of incubation under those conditions which have been arbitrarily selected as a standard (Table XII).

TABLE XI.

Concentration of Solids in the Whole Egg during Incubation.

Age.	W_e	L	S	S_t	Humidity 91 per cent.				Humidity 67.5 per cent.			
					H	W_t	H_t	$\dfrac{S_t}{H_t}$	H	W_t	H_t	$\dfrac{S_t}{H_t}$
days	mg.	gm.	gm.	gm.	gm.	gm.	gm.		gm.	gm.	gm.	
0				12.86	0	52.10	39.24	32.8	0	52.10	39.24	32.8
1				12.86	0.07	52.03	39.17	32.8	0.24	51.84	38.98	33.0
2				12.86	0.13	51.97	39.11	32.9	0.48	51.62	38.76	33.2
3				12.86	0.20	51.90	39.04	32.9	0.73	51.37	38.51	33.4
4				12.86	0.26	51.84	38.98	33.0	0.97	51.13	38.27	33.6
5	221			12.86	0.33	51.77	38.91	33.0	1.21	50.89	38.03	33.8
6	423		0.01	12.85	0.40	51.70	38.85	33.1	1.45	50.65	37.80	34.0
7	735	0.01	0.02	12.85	0.46	51.65	38.80	33.1	1.69	50.42	37.57	34.2
8	1,189	0.01	0.04	12.83	0.53	51.58	38.75	33.1	1.94	50.17	37.34	34.4
9	1,879	0.02	0.07	12.81	0.59	51.53	38.72	33.1	2.18	49.94	37.13	34.5
10	2,661	0.03	0.12	12.77	0.66	51.47	38.70	33.0	2.42	49.71	36.94	34.6
11	3,750	0.04	0.18	12.72	0.73	51.41	38.69	32.9	2.66	49.48	36.76	34.6
12	5,105	0.05	0.26	12.65	0.79	51.36	38.71	32.7	2.90	49.25	36.60	34.6
13	6,839	0.07	0.36	12.57	0.86	51.31	38.74	32.5	3.14	48.06	36.46	34.5
14	8,974	0.09	0.49	12.46	0.92	51.27	38.81	32.1	3.39	48.80	36.34	34.3
15	11,460	0.11	0.66	12.31	0.99	51.22	38.91	31.6	3.63	48.58	36.27	34.0
16	14,390	0.14	0.86	12.14	1.06	51.18	38.04	31.1	3.87	48.37	36.23	33.5
17	17,950	0.18	1.09	11.95	1.19	50.09	39.14	30.5	4.36	47.92	35.97	33.2
18	22,030	0.22	1.35	11.73	1.32	50.00	39.27	29.9	4.84	47.48	35.75	33.8
19	26,670	0.27	1.60	11.53	1.45	50.92	39.39	29.3	5.32	46.05	35.52	32.5

$W = 57.8$ = weight of whole egg (average 500 + eggs) before incubation.

$L_0 = 5.7$ = weight of shell (9.81 $\doteq$ 0.11 per cent) before incubation.

$W_0 = 52.1$ = weight of egg contents before incubation.

W_t = weight of egg contents after t days of incubation = $W_0 - H$.

$H_0 = 39.24$ = weight of water (75.3 per cent) before incubation.

H_t = weight of water after t days of incubation = $W_t - S_t$.

$S_0 = 12.86$ = weight of solids (24.7 per cent) before incubation.

S_t = weight of solids after t days of incubation = $S_0 - S + L$.

S = weight of solids burned (*cf.* Column 4, Table X).

$W_e = \dfrac{t^{3.6}}{1.496}$ = weight of embryo (mg.) (*cf.* Column 1, Table X).

$L = 0.01 W_e = \dfrac{t^{3.6}}{0.1496}$ = weight of solids added to the egg contents from the shell (gm.).

$H = 0.0075 (100 - h) t$ = water lost by evaporation (0 — 16 days).

$H = 0.0075 (100 - h) t + 0.0075 (100 - h) (t - 16)$ = water lost by evaporation (17 — 19 days).

$\dfrac{S_t}{H_t}$ = solids (gm. per 100 gm. H_2O) after t days of incubation.

TABLE XII.

Solid, Fat, Nitrogen, and Water in Egg Contents during Incubation under Standard Conditions.

Age.	(1) Solids $= S_t$	(2) Water $= H_t$	(3) Fat $= F_t$	(4) Nitrogen $= N_t$	(5) Per cent $N = \dfrac{N_t}{S_i}$	(6) C_t
days	gm.	gm.	gm.	gm.	per cent	
0	12.86	39.24	5.79	0.974	7.57	1.00
1	12.86	38.98	5.79	0.974	7.57	1.00
2	12.86	38.76	5.79	0.974	7.57	1.00
3	12.86	38.51	5.79	0.974	7.57	1.00
4	12.86	38.27	5.79	0.974	7.57	1.00
5	12.86	38.03	5.79	0.974	7.57	1.00
6	12.85	37.80	5.78	0.974	7.58	1.00
7	12.85	37.57	5.77	0.974	7.58	1.00
8	12.83	37.34	5.75	0.974	7.59	1.00
9	12.81	37.13	5.72	0.974	7.60	1.00
10	12.77	36.94	5.67	0.974	7.62	1.01
11	12.72	36.76	5.61	0.974	7.66	1.01
12	12.65	36.60	5.53	0.974	7.70	1.02
13	12.57	36.46	5.43	0.974	7.75	1.02
14	12.46	36.24	5.30	0.974	7.81	1.03
15	12.31	36.27	5.13	0.974	7.91	1.04
16	12.14	36.23	4.93	0.974	8.02	1.06
17	11.95	35.97	4.70	0.974	8.15	1.08
18	11.73	35.75	4.44	0.974	8.30	1.10
19	11.53	35.52	4.19	0.974	8.45	1.12

Columns 1 and 2 from Table XI.

Column 3, $(F_0 = 0.45\ S_0 = 5.79$ gm. (Table VI); $F_t = 5.79 - S$ (Column 4, Table X). The initial concentrations for fat and nitrogen are derived from Table VI.

Column 6, $C_t =$ the constant at t days, by which the initial preincubation concentration of any substance (if expressed as per cent of dry solid) must be multiplied to find the normal curve, which may be used as a base line for comparison with the values actually obtained. The reason for this is that due to the continuous loss of oxidizable solid during incubation the concentration of any substance, if unaffected during incubation, will nevertheless appear to rise, since the total amount of solid substance is falling.
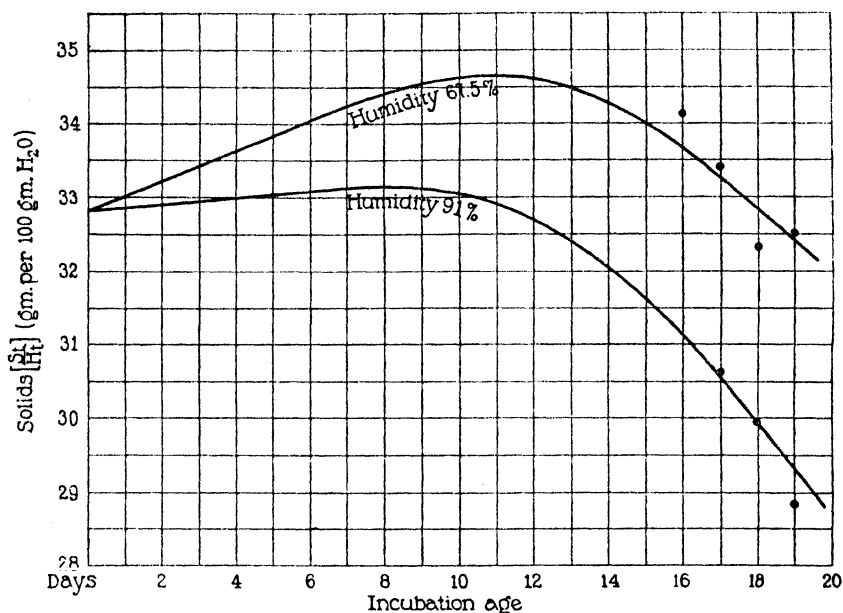
FIG. 9. Theoretical curves to indicate the changing concentration of solids (gm. per 100 gm. H_2O) in the whole egg during the incubation period at humidities of 67.5 and 91 per cent (Table XI). The black circles show the values actually obtained (Table VII) in eggs incubated at these humidites (corrected from 65 and 90 per cent respectively, according to the graph (Fig. 4).

DISCUSSION AND SUMMARY.

As this paper goes to press a complete review of the chemistry of the fertile egg will be appearing (19). The author, Mr. J. Needham, was kind enough to allow me to inspect his manuscript and thus avail myself of the comprehensive bibliography and discussion. It is surprising that no biochemists have estimated the changing water content of the egg during incubation. Many of the analyses reported in Needham's review were expressed in per cent of total weight or per cent of dry solid, and consequently are of questionable value, since these latter functions are themselves changing; the former due to water evaporation and the latter through the addition of shell constituents and the burning of oxidizable organic compounds. Moreover, there has been no statistical treatment of the results, and the reliability of

the average figures obtained has consequently been difficult to estimate.

.Tangl's work, quoted throughout this paper, except for its lack of statistical treatment is more enlightening. However, his concept of the so called "Energy of Embryogenesis" which he propounds, seems to me misleading and unwarranted. What Tangl measured was the amount and the caloric value of the solid material burned and thus the quantity of energy lost during the embryonic period. The latter is equivalent to the usual measurements of catabolism. In the case of the embryo it is not basal metabolism which is being estimated, since the conditions are not basal. The embryo is absorbing and assimilating nutriment all the while at a relatively rapid rate.

The calorific value of the oxidized solid, which is in truth the amount of energy lost during a certain chosen interval, in Tangl's judgment stands for the energy of embryogenesis; i.e., the energy of development (growth + differentiation). We believe that this conception is erroneous. The two processes, anabolism and catabolism, occur together and undoubtedly have some relationship, but surely one is not a measure of the other.

In a starving animal, and so probably in a starving embryo, there is a considerable amount of so called basal metabolism. Thus if the "Embryogenetic Energy" were measured under these conditions a figure would be obtained for which there was no growth to correspond, or in other words there would be a value for something which did not exist.

It will be seen in our later communications that the changes with age of metabolic rate and growth rate do not coincide. The amount of catabolism under certain circumstances does not accelerate growth or anabolism, but seems rather to be a limiting factor. It is as if when the absorbed energy were constant an increase of catabolism would .make inroads upon the amount of energy which otherwise would remain for storage (growth).

If, as Pembrey's (20) experiments would tend to show, there is an increase of metabolism in the oldest embryos when the outside temperature is lowered, one would find at the end of incubation in such cases that there was a *greater* amount of so called "Energy of Development" but *smaller* embryo. It seems that the potential energy

amassed as growth comes from that remaining after the needs of the body have been satisfied.

The results of the experiments described in this paper have formed the basis for judgment in the selection of suitable standard conditions for the incubation of hen's eggs. Standardization was necessary so that in future experiments the more important environmental factors might be kept uniform within a certain appropriate range and therefore not be held accountable for deviations observed in the embryos.

Henceforth in this series of papers the term "standard incubation conditions" will signify that (1) the temperature was constantly at $38.8 \pm 0.4°C.$, (2) the humidity at 67.5 ± 2.5 per cent, (3) there was a continuous flow of warm air into the incubator to provide the necessary circulation, and (4) the eggs were rolled once a day within the constant temperature room.

The incubator, a double-walled copper cabinet, stands in a constant temperature room, the fluctuations of which are $\pm 1.0°C.$ The space between the walls of the incubator is filled with water which serves as a buffer to outer variations.

It might be repeated that all the eggs are from White Leghorn hens, are incubated 2 days after laying, and that they are kept cold during the interval necessary for transportation.

With the figures from our chemical analyses and metabolic rate experiments, it was possible to calculate values for the concentration of total solids, fat, and nitrogen throughout the incubation period. These data were necessary as a general chemical background for further work. The results of the calculations are obviously rough. Because of the great variability of the eggs a satisfactory degree of accuracy could not have been attained without a very large number of analyses supplemented by complete statistical treatment. The necessity for such a comprehensive study was not evident, and it is our belief that the approximations reached in this paper are sufficiently close to serve our present purposes.

The chief facts that have been ascertained in this investigation are

(1) Loss of water by the egg during incubation is a function of the atmospheric humidity in its immediate environment. More rapid circulation of air lowers the humidity around the egg and thus increases

evaporation.   Other facts influencing evaporation are (a) atmospheric temperature, (b) thickness and surface area of the shell, and (c) conditions within the egg, the most important of which, it is suggested, is the amount of heat produced by the embryo.   The latter factor, in turn, depends upon its size and age, and a significant change does not become apparent until the last 3 or 4 days of incubation, that is to say, when the embryo is of sufficient mass to exert a measurable force.

(2) The surface area of the eggs in sq. cm. may be approximately represented by the formula $S = K W^{\frac{2}{3}}$, where $K = 5.07 \pm 0.10$, and W = the weight of the whole egg in gm.

(3) There is a loss of weight by the shell during incubation.   This is most noticeable near the end of the cycle, when the loss seems to parallel in general the weight of the embryo.

(4) There is also a loss of solid matter during incubation.   Chemical analyses indicate that about 98 per cent of the material oxidized is fat. This conclusion is corroborative of previous work by Hasselbalch, Hasselbalch and Bohr, and Tangl.

(5) Carbon dioxide may be measured with relative accuracy. When it is assumed that it is derived from the oxidation of fat, satisfactory corroboration of the chemical analyses is obtained.

These experiments have furnished the data from which the values have been calculated for total solids, fats, and protein in the whole egg throughout incubation.   The figures may be used later for comparison with the concentration of these substances within the embryo.

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PHYSIOLOGICAL ONTOGENY.

A. Chicken Embryos.

III. Weight and Growth Rate as Functions of Age.

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Growth and division of cells represent one of the characteristic signs of life. In an analysis of the phenomenon of ontogenesis the general growth rate as estimated by the increase in mass must be included as one of its most striking and significant features. The present investigation concerns itself with the weight and growth rate of chicken embryos as functions of age. The source of the eggs and the management of incubation have been described in a previous paper (1).

Method.

Fertile eggs of from 5 to 19 days of incubation age were opened. After cutting the membranes the embryos were removed and separated at the distal end of the yolk stalk. Various methods were used in an attempt to prevent loss of blood from the vessels or retention of an undue amount of amniotic moisture on the skin. Laying the embryos momentarily on a piece of filter paper or on a smooth towel, or passing a current of air over their surfaces was tried. Occasionally the vessels were clamped. We were never confident of the accuracy of certain points in our technique in weighing the smaller embryos, such for instance as whether the water adhering to the skin compensated for the frequent loss of a drop or two of blood. In the older embryos only did we feel assured that the errors incurred from these sources were minimal. To include the amniotic and chorionic membranes, which would have made an appreciable difference in the weight was

deemed desirable from the theoretical consideration that these structures are composed of living cells, presumably sharing in the general metabolic activity of the body.   To do this, however, without the loss of blood and without including other non-functioning parts and adherent water was found to be impractical.   In this and subsequent researches then we have concerned ourselves only with the embryo itself, assuming that the error incurred by neglecting the membranes was either constant throughout incubation or negligible.   In consequence, although the factors analysed, such as growth rate, metabolism, and chemical constitution, may be compared one with another they may have only an approximate general validity due to this omission.   In some of the 19 day old embryos the yolk sac, which could not properly be considered a part of the living organism, had been taken up into the abdominal cavity, and so in such cases it was necessary to squeeze it out gently and separate it before weighing the chick.

The embryos were weighed in weighing bottles.   With the younger chicks, when certain chemical analyses were to be made, a number of embryos were placed in the same bottle so that the variations of the individual weights for these ages could not be obtained.   They were probably considerable.

Most of the embryos included in this particular investigation developed in a Lo-Glo incubator, that is to say before conditions had been standardized as outlined in the preceding communication.   In this incubator the temperature conditions were not perfectly uniform, so that some eggs probably developed faster than others due to their position in the drawers.   No provision was made for this discrepancy except that the eggs were rolled each day, the drawers frequently changed, and the weighings continued over an 8 months period so that the average value for any one age does not represent a particular group of embryos subjected to particular conditions.   After the completion of this series, when a constant temperature room for our experiments had been constructed, we were able to make a number of weighings under standard temperature and humidity conditions for comparison.

The sex was not determined.   The results, therefore, represent a mean average of the weights of White Leghorn embryos varying from

5 to 19 days of age and incubated at a temperature of about 39°C., and a humidity of about 70 per cent. It was hoped that with a rather large number of weighings the chances for the equalization of factors causing deviations from the mean, namely, of temperature, humidity, sex, and technical errors would be optimal.

TABLE I.

Weight, Growth Rate, and Acceleration of Growth of Chicken Embryos.

Age.	Log age.	No. of embryos.	Mean wet weight and probable error.*	Log wet weight.	$\frac{\text{Log } W = 3.6}{\text{Log } t - 0.175}$ Wet weight from formula.	Log wet weight from formula.	Percentage growth rate. $\frac{dw}{dt}$ $\overline{W}$ $= \frac{3.6}{t}$	Acceleration. $\frac{d\left[\frac{dw}{dt}\frac{1}{W}\right]}{dt} = -\frac{3.6}{t^2}$
days			*mg.*		*mg.*			
5	0.699	200	206	2.314	221	2.345	0.720	−0.1440
6	0.778	45	424	2.627	423	2.626	0.600	−0.1000
7	0.845	91	730	2.863	735	2.866	0.514	−0.0735
8	0.903	42	1,200 ± 19	3.079	1,189	3.075	0.450	−0.0562
9	0.954	48	1,916 ± 163	3.282	1,817	3.259	0.400	−0.0445
10	1.00	48	2,614 ± 23	3.417	2,661	3.425	0.360	−0.0360
11	1.041	27	3,738 ± 62	3.572	3,750	3.574	0.327	−0.0298
12	1.079	15	5,010 ± 116	3.700	5,105	3.708	0.300	−0.0250
13	1.114	35	7,239 ± 120	3.859	6,835	3.835	0.277	−0.0213
14	1.146	15	9,484 ± 220	3.977	8,974	3.953	0.257	−0.0184
15	1.176	12	11,734 ± 322	4.069	11,460	4.059	0.240	−0.0160
16	1.204	10	14,343 ± 242	4.156	14,390	4.158	0.225	−0.0141
17	1.230	35	18,364 ± 215	4.264	17,950	4.254	0.212	−0.0125
18	1.255	29	20,800 ± 334	4.318	22,030	4.343	0.200	−0.0111
19	1.278	30	26,341 ± 411	4.421	26,670	4.426	0.190	−0.0100

*This is the usual probable error of the biometrician; *i.e.*,

$$0.6745 \frac{\text{standard deviation}}{\sqrt{\text{number of observations}}}.$$

RESULTS.

The results have been summarized in Table I. Curves of the average weights (Fig. 1) and of their logarithms (Fig. 2) have been plotted as functions of age. The latter, which is comparable to percentage weight increments, shows by its slope that the greatest relative changes in weight occur in the early days. The percentage increase in mass rather than the actual increments of weight is taken

as the rate of growth because this aspect is considered biologically more significant.   Either definition is permissible, but from a functional standpoint it would seem that the object of interest is the growth and divisional rate per unit mass.   When the weight increments are taken as the basis of growth rate, the quantity of tissue taking part in the reaction is left out of consideration.   For instance, if an embryo
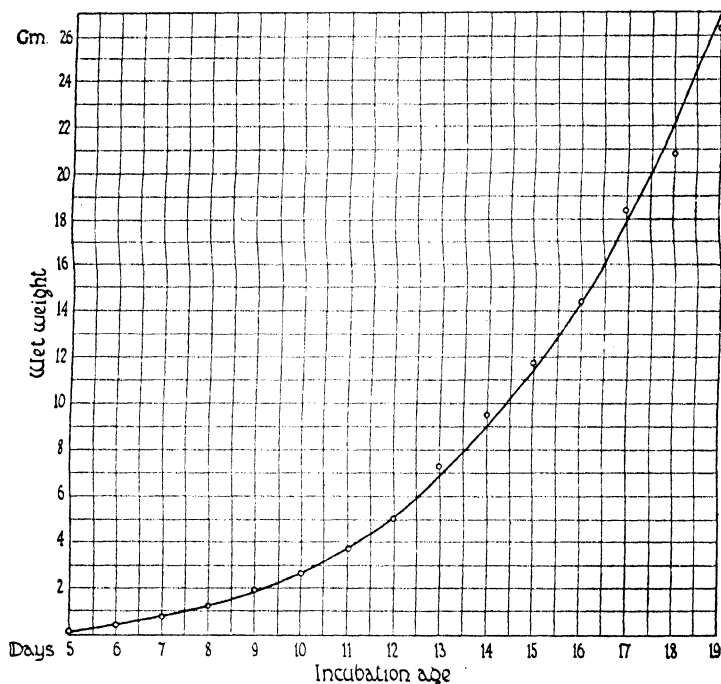
FIG. 1. To show the average wet weight of chicken embryos as a function of age (Table I).

weighing approximately 2.5 gm. is found to have gained 1.0 gm. in 24 hours its growth rate, using increments as the criterion, would be considered equal to that of a chicken weighing, let us say, 50.0 gm. which also gained 1.0 gm. in the same period.   From a physiological standpoint this conception of the rate of growth is misleading, and does not allow for a comparison between the same organism at different ages or organisms of different species at the same age.   Henceforth
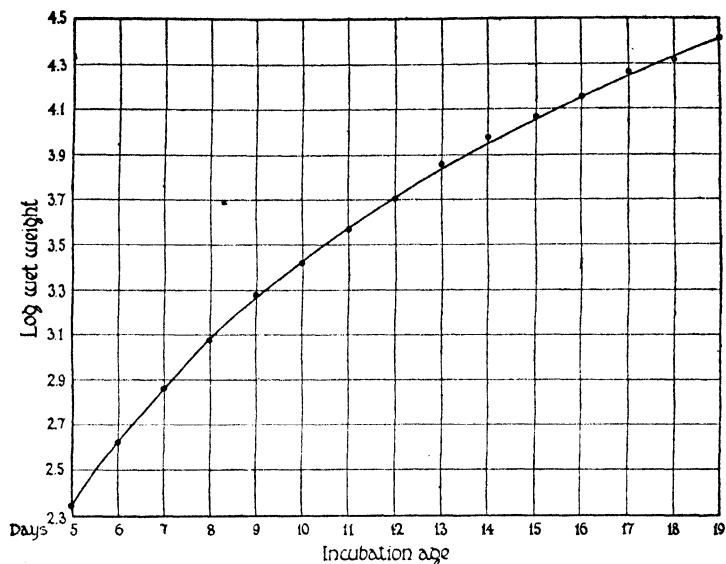
FIG. 2. The logarithm of the average wet weight equated against the age of the embryo (Table I).
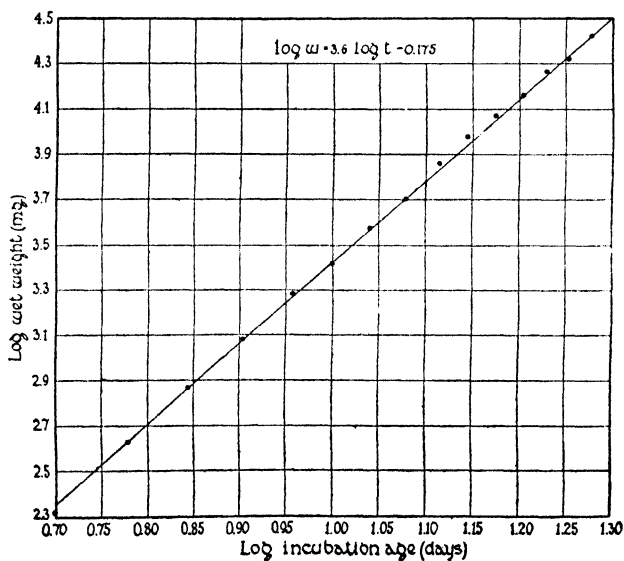
FIG. 3. The logarithm of the average weight expressed as a function of the logarithm of the incubation age in days (Table I).

when the term growth rate is used, we shall mean the percentage rate
of increase in mass; *i.e.*, velocity $= v = \dfrac{\frac{dw}{dt}}{W} = \dfrac{d}{dt}\left[\dfrac{dw}{W}\right] = \dfrac{1}{W} \cdot \dfrac{dw}{dt}$.

It was found that when log W was equated against log t the points
approximated 'a straight line (Fig. 3).   The formula log W = 3.6
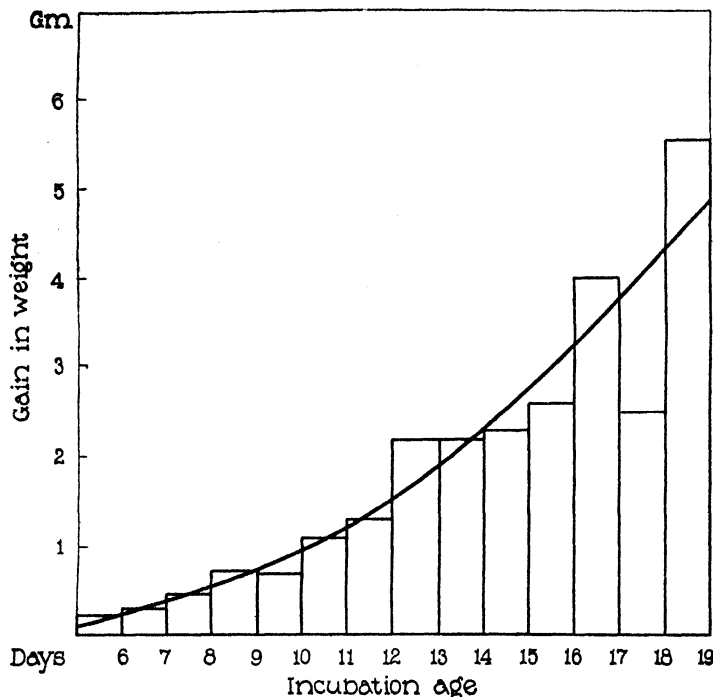
FIG. 4. The increments of growth for each day of incubation.   The curve is the
theoretical expression for the increments as derived from the formula.

$$W = 0.668t^{3.6}$$

log t − 0.175 was obtained graphically.   By its use the graphs in all
the figures were drawn.   These figures are included in the table
(Table I) so as to allow for comparison with the average findings.
We do not attach theoretical significance to the fact that the weights
can be expressed by such a simple equation.   If certain intervals of
growth are chosen, and particularly if these are sufficiently short,

it is not difficult to find simple equations to fit weight figures. Various mathematical expressions have recently been suggested to conceptualize the growth process. These may be useful devices but they do not furnish, in our judgment, sufficient evidence to establish the nature of the "master reaction" of growth (2). The embryonic period may be considered as one, or the major part of one, of the three chief cycles, or periods of accelerated growth, in the life span. The rate slows asymptotically as the chick approaches an equilibrium with its immediate environment before hatching. When the increments of
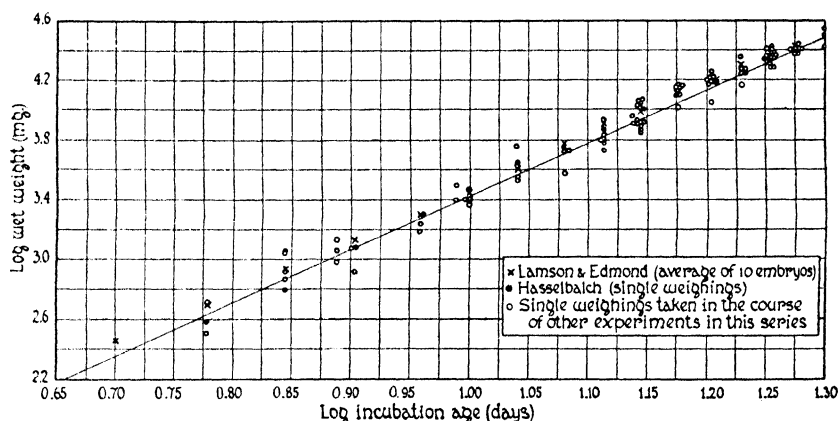
FIG. 5. The graph obtained from the formula log $W = 3.6$ log $t - 0.175$ compared to the average results obtained by Lamson and Edmond ($\times$) the single weighings of Hasselbalch ($\bullet$) and some additional embryonic weighings made in the course of later experiments ($\circ$).

growth are represented graphically (Fig. 4) it is seen that except for the 17 to 18 day interval there is a rather gradual smooth ascent in the values. Later weighings for 18 day (log 18 = 1.255) old embryos, as shown in Fig. 5, indicate that the average weight at this age is probably higher than was first estimated and that the break in the curve does not represent the usual occurrence. It is suggested that Brody's rhythmic growth curves (3) for the embryonic period were due to similar chance variations, since they do not coincide one with another, and since the figures on which they were based were obtained

as a result of a very small (Lamson and Edmond (4)) or a minimal (Hasselbalch (5)) number of weighings.

Single weighings after incubation conditions had become standardized by the construction of a temperature room as previously described are shown in Fig. 5 for comparison with the standard curve (Fig. 3). To these are added the figures collected by Brody (3) after Lamson and Edmond, and Hasselbalch.

By the use of the formula the percentage rate of growth may be obtained.

$$\frac{d}{dt}\left[\frac{dw}{W}\right] = \frac{K}{t}$$
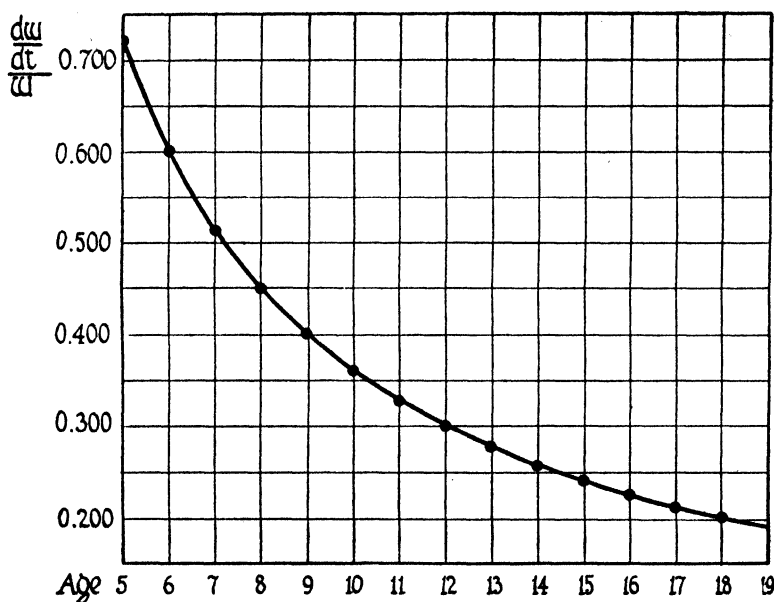
FIG. 6. The percentage growth rate as a function of age (Table I).

where $K = 3.6$. The rate of growth as given by this equation may then be plotted as a function of age (Fig. 6). It is seen that the percentage rate of growth decreases progressively with age. If the rate of growth is used as a criterion of age the velocity of aging (senescence), or in other words, the negative acceleration of the percentage

increase in weight with time (Fig. 7), may be obtained by a further differentiation:

$$\frac{d}{dt}\left[\frac{\frac{dw}{W}}{dt}\right] = -\frac{K}{t^2}$$

where $K = 3.6$.

Minot's dicta were that (1) the rate of growth depends on the degree of senescence; (2) senescence is at its maximum in the
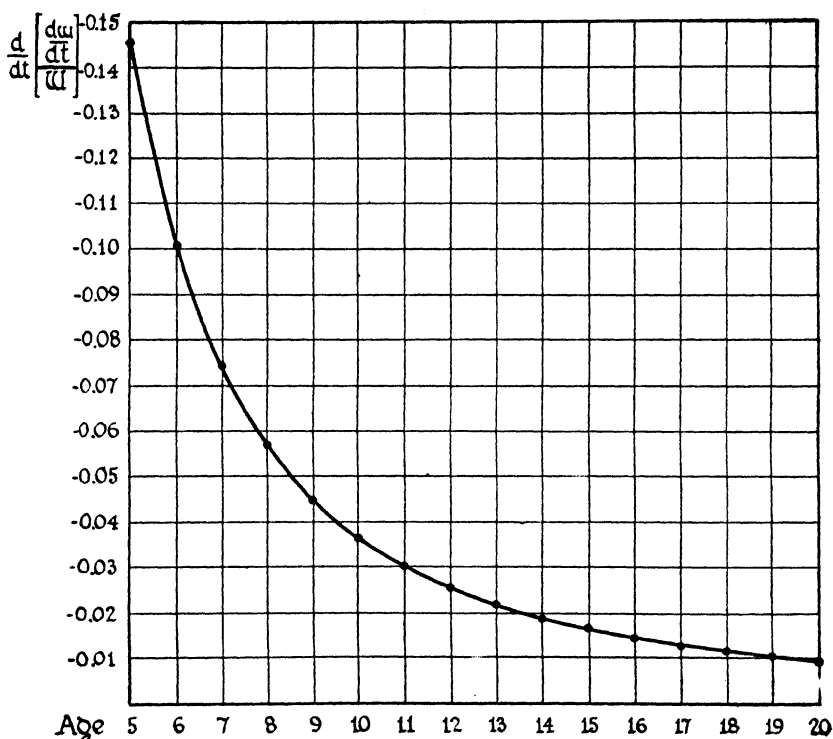
FIG. 7. The negative acceleration of growth as a function of age (Table I).

very young stages; (3) the rate of senescence diminishes with age; and (4) as a corollary from these, natural death is the consequence of cellular differentiation (6). We cannot at present accept this statement as a satisfactory description of senescence, for there is yet no evidence to show that the degree of "aliveness" and velocity of

growth are synonymous.   There are other and perhaps more significant phenomena than growth rate which change with age.   An organism as a force can only be interpreted in terms of totality of function.   Minot's concept that death is the consequence of cellular differentiation seems to be at variance with his other hypothesis. For instance, tissue differentiation, as far as can be judged from its chemical constitution, occurs most rapidly during the latter part of the embryonic period rather than at the beginning.   This phase of the subject will be discussed in a later paper.

SUMMARY.

1. The average weights of chicken embryos between 5 and 19 days of incubation as found by over 600 weighings may be expressed by a simple exponential equation,

$$W = K\,t^{3.6}$$

where $K = 0.668$.

2. The velocity of growth (*i.e.* the percentage increase in mass) is inversely proportional to the incubation age.   The product of the two (vt) is a constant (3.6).   The negative acceleration of growth likewise decreases with age.

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TEMPERATURE CHARACTERISTIC FOR LOCOMOTOR ACTIVITY IN TENT CATERPILLARS.

BY W. J. CROZIER AND T. B. STIER.

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I.

At different temperatures young tent caterpillars (*Malacosoma americanum*) were caused to creep vertically upward upon a thin wooden rod (3 mm. diameter) with evenly roughened surface. The rod was 24 cm. long, and the larva began ascent at the bottom; the mid-region of the rod was graduated, so that the time taken to travel 10 cm. could be accurately observed. Simultaneously, the number of "peristaltic" locomotor waves required to cover the 10 cm. distance was obtained by counting the steps of the anal prolegs. In case the initial or the terminal step failed to coincide precisely with the selected graduation mark, an estimate was made of the fractional step involved. The rod and a thermometer were suspended in a large box which also contained an electric heating device controlled with a rheostat. The box was set up in a room having approximately the desired temperature. For higher temperatures a small dark room was used which could be heated electrically. The animals were adapted to the temperature of the experiment until the rate of creeping became constant.

From the observations taken it was possible to compute: (1) the speed of progression; (2) the frequency of abdominal locomotor waves; (3) the mean amplitude of the step taken by the anal prolegs. We desired to consider these quantities in their relations to temperature, and to compare the critical thermal increment of locomotor activity with that found for some homologous activities.

It was found that variation in speed of creeping could be adequately controlled by the temperature, provided animals of the same size were used. Among such individuals, especially if taken from the

same nest, the rate of creeping is very uniform. We have used caterpillars 1.5 to 2.0 cm. long. About 14 individuals were employed in obtaining 180 measurements. The points plotted in Fig. 3 are each the average of 6 or more very closely concordant determinations.

II.

When the mean speeds of creeping at different constant temperatures are considered in terms of the relationship known to be valid for a number of similar instances,

$$\log_e \frac{\text{velocity at } T_2}{\text{velocity at } T_1} = \frac{\mu}{2}\left(\frac{1}{T_1} - \frac{1}{T_2}\right),$$
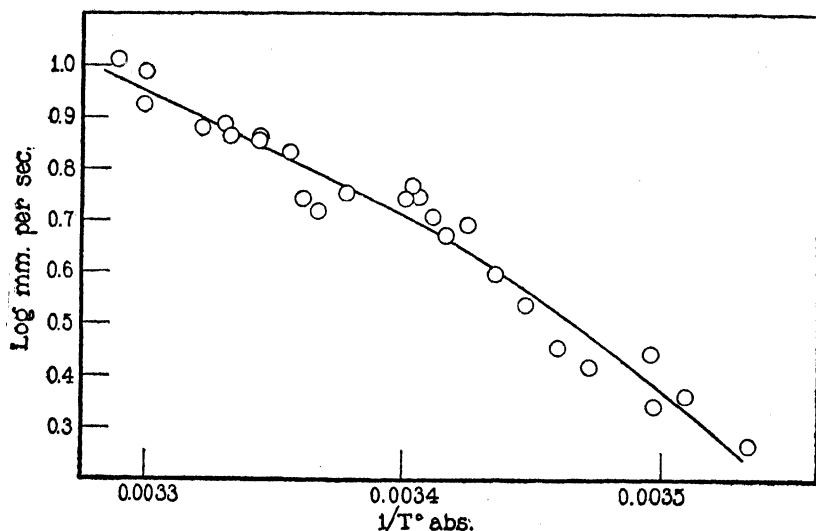
FIG. 1. Mean velocity of creeping is plotted logarithmically against reciprocal of absolute temperature. The relationship is not rectilinear.

the graphs of log velocity *versus* 1/absolute temperature fails to be rectilinear (Fig. 1). No significant value of the critical increment μ can be calculated.

This result finds its explanation in the fact that one of the two components determining the speed of vertical ascension is influenced by the temperature in a special way. The act of creeping is begun by a forward movement of the anal prolegs, initiating a peristaltic

body wave coursing cephalad to the anterior margin of the abdomen; simultaneously the anterior thoracic legs begin their progression movements, the wave of leg activity running posteriorly. The abdominal movements may be timed by counting the steps of the anal prolegs. Between 20° and 30° the number of such steps per 10 cm. distance is sensibly constant. Above 30° there is a tendency for the amplitude of the steps to become less, but the available observations are not numerous because creeping is so often irregular at these higher temperatures. Below 20°, however, the amplitude
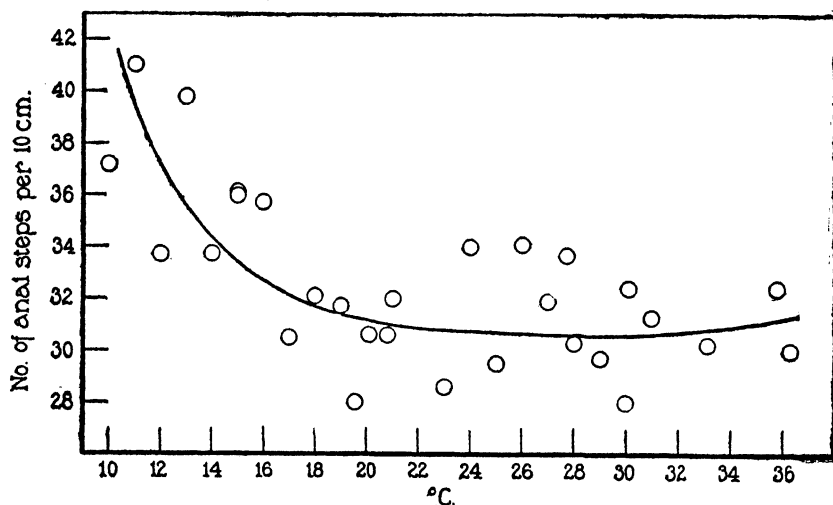
Fig. 2. The number of proleg steps required to ascend 10 cm. is increased as the temperature falls, below 20°.

decreases markedly and regularly as the temperature falls (Fig. 2). It is clear that as a consequence the value of the product (*amplitude of step*) × (*frequency of steps*), which is the velocity of progression, will be abnormally lowered at temperatures less than 20° ± (*cf.* Fig. 1).

The speed of creeping is thus deprived of its possible analytical utility, and it becomes necessary to deal directly with the *frequency* of the locomotor steps. The shortening of the amplitude of the locomotor wave at lower temperatures may have a physical basis in the physiology of the caterpillar's musculature, or it may be condi-

tioned by the vertical attitude in these experiments. Tests with horizontal creeping suggest the probability of the former alternative. It is in any case not an artifact due to irregularities of creeping; all instances in which the larva "hesitated" were excluded.

The frequency of the locomotor steps, on the other hand, is very definitely related to the temperature by the Arrhenius equation (Fig. 3). The temperature characteristic, μ, has the value 12,200 ± 100 calories. At temperatures below 11° the frequency tends to
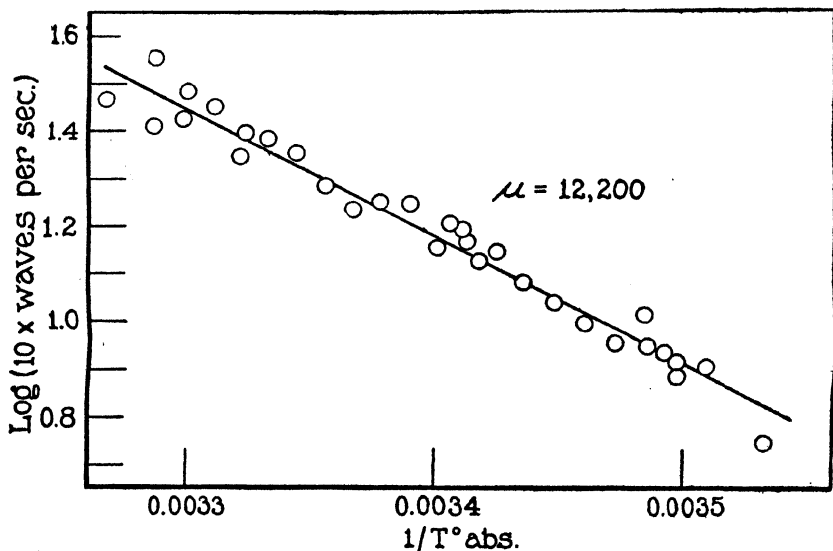
FIG. 3. The frequency of anal proleg steps (F) is related to the temperature by the equation $\log F = \dfrac{-\mu}{2T} + C$, where T is the absolute temperature.

be abnormally low, and above 30° (a "critical point" in experiments with other insects also (cf. Crozier and Stier, 1924–25)) many larvæ do not creep continuously. In a few cases regular locomotion was obtained at temperatures as high as 36°C., but the mean frequencies of steps are always lower at these temperatures than the relation given by Fig. 3 would demand.

III.

Various rhythmic neuromuscular activities of arthropods consistently exhibit the temperature characteristic 12,200 (Crozier,

1924–25, a), with which that now obtained for frequency of loco-
motor movements in tent caterpillars shows satisfactory agreement.
Abdominal respiratory movements must be excluded from this
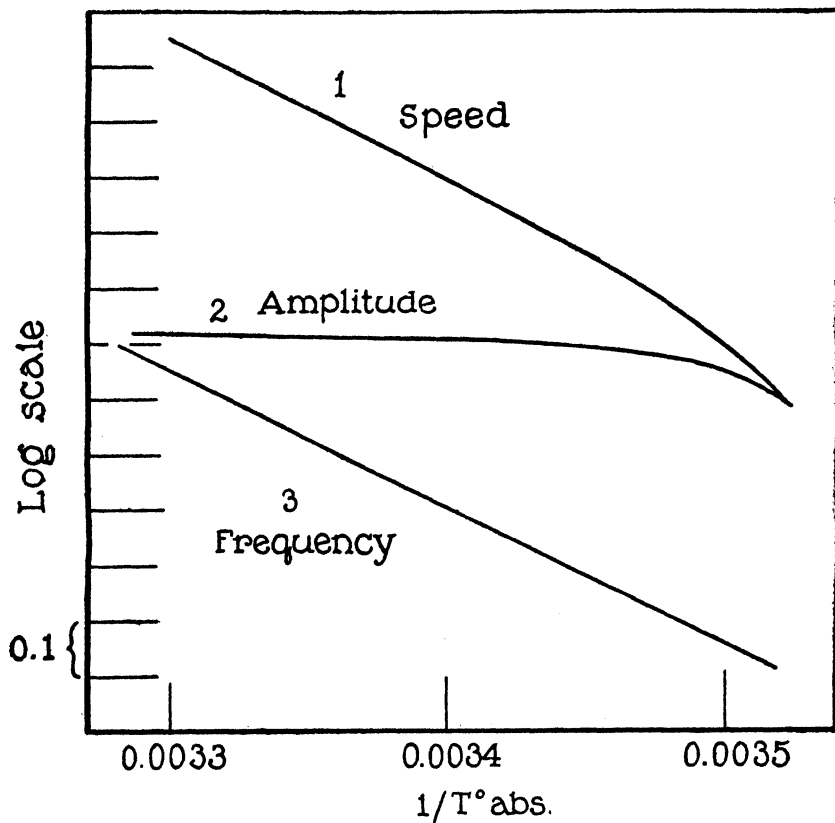
FIG. 4. The speed of vertical ascension at any temperature = frequency of
steps × mean amplitude of step. The curves for *speed* and for *amplitude* are
respectively taken directly from the smoothed curves in Figs. 1 and 2, that for
frequency from Fig. 3. (One ordinate division = 0.1 on log scale.) Curve 1 is
also the resultant of adding the corresponding ordinates of Curves 2 and 3.

category (Crozier and Stier, 1924–25). The coincidence of the
present finding with the result in other instances involving activities
of arthropods presumably controlled by nerve center discharge sub-
stantiates the view that homologous activities may be governed in

chemically similar, or even identical, ways; and that from this standpoint the classification of quantitative aspects of behavior is possible upon the basis of the respective critical increments.

The locomotion of the tent caterpillar is especially interesting because it shows how the analysis of a case in which a velocity (*i.e.* of movement) fails to be a simple exponential function of $1/T^{\circ}_{abs.}$ is readily rectified when the mechanism of the process is examined with sufficient detail. Curves of the general type exemplified by Fig. 1 have been indicated (Crozier, 1924–25, *b*) as resulting when competing processes have a cumulative effect upon the observed velocity. The creeping of *Malacosoma* provides a kind of model illustrating this conception (*cf.* Fig. 4).

SUMMARY.

The frequency of abdominal peristaltic locomotor waves in tent caterpillars during vertical ascension is controlled by the temperature according to the equation of Arrhenius. The constant μ (temperature characteristic) has the value 12,200 calories, agreeing quantitatively with the value obtained for a number of other (non-respiratory) rhythmic neuromuscular movements among arthropods.

CITATIONS.

Crozier, W. J., 1924–25, *a, J. Gen. Physiol.*, vii, 123.
Crozier, W. J., 1924–25, *b, J. Gen. Physiol.*, vii, 189.
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THE EFFECT OF X-RAYS ON THE IRRITABILITY OF MUS-CLES IN THE FROG.

By IRVING KUSHNER.

(From the Laboratory of Physiology, College of the City of New York, New York.)

INTRODUCTION.

In view of the fact that x-rays are at the present time largely used in clinical practice, it seems advisable to investigate the action of the x-rays upon some of the more simple animal processes. Of these, the response of a single muscle to a single electrical stimulus is probably the simplest and best known, and for this reason was chosen for the present research.

Methods.

The usual apparatus for obtaining graphic records of contractions was employed, but certain modifications were found necessary.

(*1*) *Muscle Preparation.*—Since the research is essentially one of comparison between an x-rayed and a normal muscle, it is clear that behavior of the normal muscle is of prime importance, and therefore that the difference between the normal and the experimental muscles should be minimal. For this reason, it was determined to employ as a control the fellow muscle preparation from the other leg of the same frog in every case. Even under these circumstances, it was soon found that differences exist between the two legs. Attempts were made to reduce these differences in various ways, on the assumption that section of the sciatic nerve first cut induces a reflex block in the other nerve. All attempts were abandoned in favor of cutting both nerves simultaneously at their entrance into the gastrocnemii muscles. This method gave a more uniform result in that the differences between the two muscle preparations were less than by any other method, and in that it was impossible to forecast which of the two muscles would react more easily.

(2) *Mode of Stimulation.*—The primary current was obtained from four dry cells. The DuBois-Reymond apparatus was used, apply-
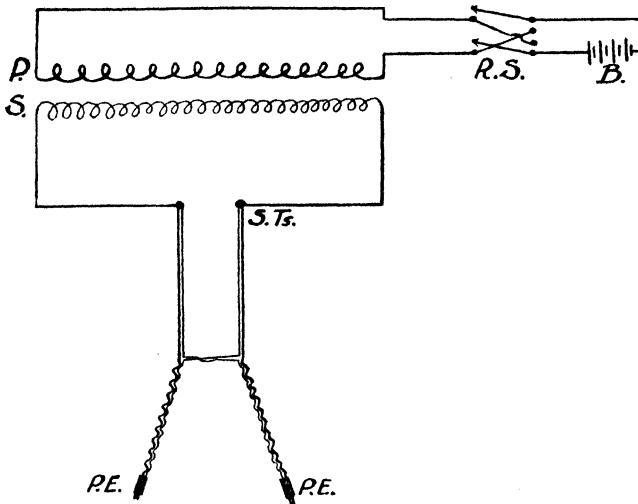
FIG. 1, *A*. DuBois-Reymond induction apparatus. *R. S.*, reversible switch; *B.*, batteries; *P.*, primary coil; *S.*, secondary coil; *S. Ts.*, second terminals; *P. E.*, platinum electrode.
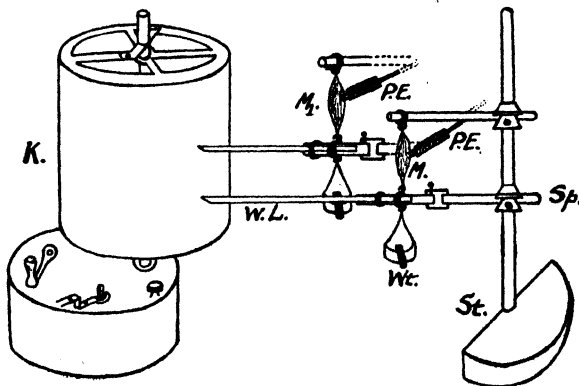
FIG. 1, *B. K.*, kymograph; *W. L.*, writing lever; *St.*, stand; *Sp.*, support; *M.-M*$_1$., muscle preparation; *P. E.*, platinum electrode; *Wt.*, weight.

ing the make and break shock. The electrodes used, were in all instances platinum.

For the purpose of the research, it was important that the stimulus applied to each member of the pair of muscles in any experiment should be the same on any given occasion and the following method was used by means of which both muscles could be stimulated simultaneously.

Attached to the secondary terminals[1] of the inductorium, were two pairs of wires (of equal length, resistance, etc.) with platinum electrodes at their free ends. The purpose was to have as nearly equal as possible the current from the secondary between the two pairs of electrodes. Each muscle was suspended from a clamp and attached to a writing lever which was counterpoised by a weight of 20 gm. In each case one of the electrodes was inserted through the muscle near the attachment of the femur, and the other, inserted near the attachment of the tendon of Achilles (Fig. 1, A, B). It is clear that under the conditions described both normal and exposed muscles received nearly equal stimuli.

In forming an idea of the condition of irritability of the muscle preparation, use was made of the observations on the minimal stimulus necessary to call forth the smallest certainly visible contraction of the muscle. In the case of a muscle in good condition, this was invariably a vigorous contraction sufficient to record itself well upon the drum: with a stimulus less than this (*i.e.* with the secondary .5 cm. farther away from the primary) not the slightest trace of a contraction even in the isolated fibrils of the muscle was discernible. On the other hand, toward the end of an experiment lasting several hours, under ordinary air conditions, it was generally possible to observe definite muscular twitches under single stimuli over a range of perhaps 3 cm. greater distance of the secondary from the primary than was necessary to afford a contraction sufficiently marked to be recorded upon the drum.

The method adopted in an experiment was as follows: ˙ The secondary was run out, after everything was ready, to a point 40 cm. at which it was anticipated that depressing the switch in the primary circuit would fail to be followed by contractions in either muscle. The coil was then moved up a half cm. at a time until either one or both muscles contracted visibly. Then the coil was moved back-

[1] Foster, M., History of physiology, London, 1890, 287.

wards in half cm. stages until contraction ceased. This point was determined for both muscles. The point on the cm. scale farthest from the primary at which a muscle just visibly contracts is the "minimal stimulus" point for that muscle for that particular time, *i.e.* the current produced by a single induction shock when the secondary
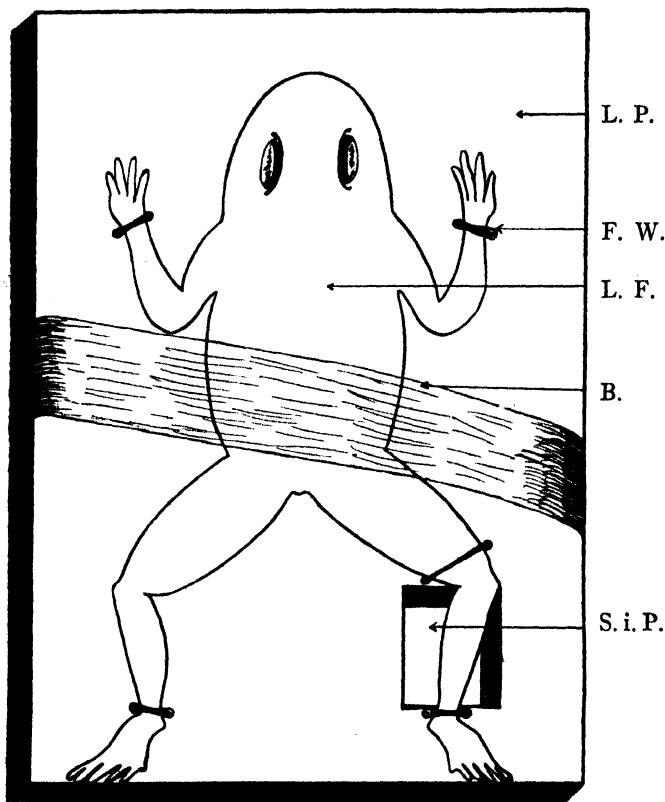
FIG. 2. *L. P.*, lead plate; *F. W.*, fastening wire; *L. F.*, living frog; *B.*, bandage; *S. i. P.*, slit in plate.

coil is at this point is the smallest electrical stimulus capable of producing a contraction in the muscle to which it has been applied. The minimal stimulus for each muscle was determined in the same way at intervals of $\frac{1}{4}$ of an hour and recorded.

(3) *X-Ray Application.*—The live frog was strapped to a lead plate[2] .25 cm. in thickness, so that one of the gastrocnemius muscles was exposed to the x-rays. This was done by strapping the animal so that one of the muscles covered a verticle slit in the lead plate, rendering it susceptible to whatever effects the x-rays might produce (Fig. 2). Both control and exposed muscles were fastened in the same manner, and extreme care was taken to serve them both equally in this respect. The plate was placed 10 inches[3] from the Coolidge tube, and the exposure was 10 milliamperes for 3 minutes from a 40,-000 volt machine with a spark gap of $2\frac{1}{2}$ inches. After exposure the frog was pithed, and the muscles prepared as mentioned above.

RESULTS OF EXPERIMENTS.

In the experiments recorded in the succeeding paragraphs, the two muscle preparations were exposed to the air after removal from the body till the end of the experiment, drying being avoided as far as possible by periodic washings with normal saline. Care was taken to treat both muscles alike in this respect. In spite of all care the various pairs of muscles did not survive the same length of time, so that a composite curve compiled from all the experiments does not afford an indication of the exact course of events that were obtained in any individual experiment. In the main, however, the two members of a pair of preparations derived from a single frog survived for approximately the same length of time.

(1) *Normal Muscle Preparation.*—In Fig. 3 is given a composite curve plotted against time of natural behavior of nine muscle preparations examined by minimal stimulus method. This is an average curve and as such approaches the results of the individual experiments.

Since, however, the composite curves obtained for the experiments in the succeeding sections are all compiled in the same way, as that for normal muscles, the minor differences between them and the individual curves may be neglected. The two fellow muscle preparations from the same frog, treated in the same way, behave in the same way as far as concerns the curves they yield in respect to successive minimal stimulation.

[2] de Courmelles, F., *Am. J. Electrotherap. and Radiol.*, 1921, xxxix, 445.

[3] Robertson, J. K., X-rays and x-ray apparatus, New York, 1924, Chapter XI.

(2) Effect of X-Ray Exposure.—In these experiments, the two fellows of the pair of preparations were subjected to identical treat-
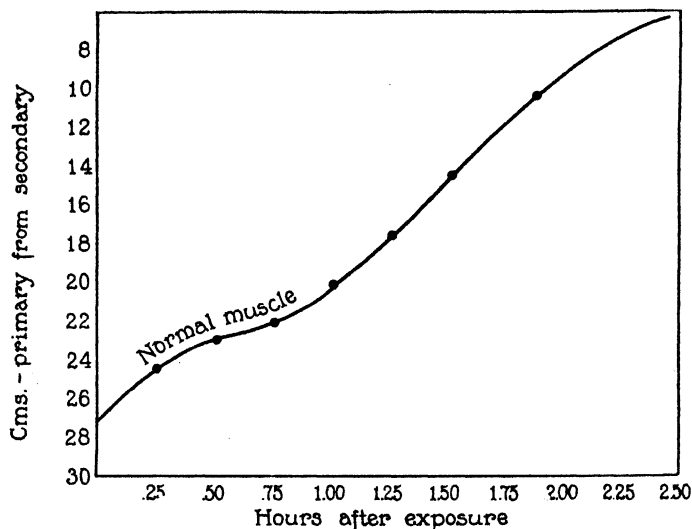
FIG. 3. Composite curve of loss of irritability of normal muscle preparations.
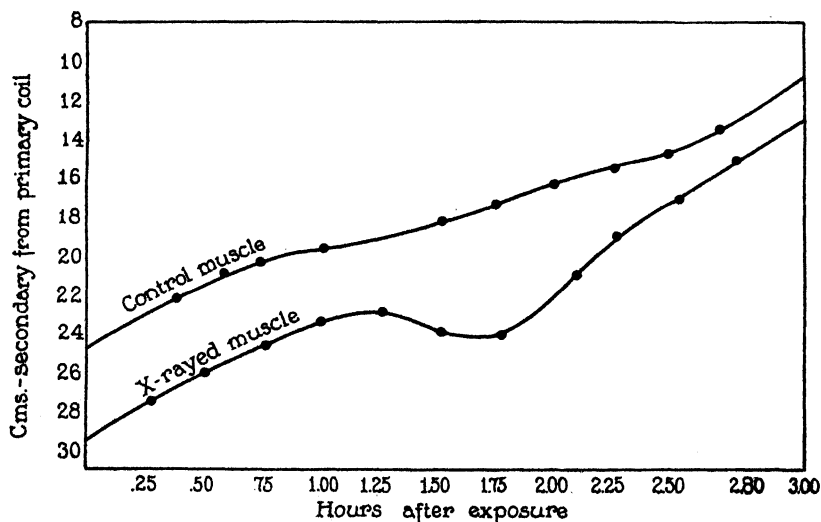
FIG. 4. Composite curves of loss of irritability of control and x-rayed muscles.

ment, except that the experimental muscle was exposed to 30 milli-ampere minutes of x-ray after the manner already described. In Fig. 4 are given the composite curves representing the means of five experiments. It is seen that the control muscles yield a composite curve of normal muscle preparations. The x-rayed muscles, on the other hand, yield a composite curve of a very different character.

During the first hour and a quarter, the curve rises like that of the control muscles, then for the next half hour it falls. From this time onward the curve rises rapidly, more rapidly it appears than the control. This behavior of the x-rayed muscles is represented with a considerable fidelity in the individual experiments, particularly the rise during the period of the first hour and a quarter and then the succeeding fall.

It is clear that the x-rayed muscle requires a smaller stimulus to call forth a visible contraction than the non-x-rayed. The pronounced difference between the x-rayed and control members of the muscle preparation is an indication that the x-rays exert a distinct influence on the muscle. It is impossible to say in what this influence consists because any decomposition of organic substances in the experimental muscle by the rays, might mean that the x-rayed muscle was being subjected to a stronger stimulus than the control. However, it appears that congestion was brought about in the experimental muscle, and what effect it produced cannot be predicted. On the other hand perhaps the increased irritability shown by the frog muscle under the action of x-rays may be a sign of degradation changes. And, from this point, the experiments do not indicate whether the action of the x-rays is to be considered as injurious or beneficial.

CONCLUSION.

Exposure of the muscle preparation of the frog to the x-rays is accompanied by a better maintenance of muscular irritability than in the case of the non-exposed preparation. This is shown by its response to a smaller electrical stimulus than the control muscle.

A COMPARISON OF BONE GROWTH IN LENGTH WITH BONE GROWTH IN WEIGHT.

By FREDERICK S. HAMMETT.

(From The Wistar Institute of Anatomy and Biology, Philadelphia.)

A comparison of bone growth in length with bone growth in weight yields information concerning the relation between growth in stature and growth in strength of supporting structure. A fairly satisfactory estimation of the relative rates, with respect to bone, of the two types of growth mentioned in earlier papers (1) is also obtained (*i.e.*, growth by increase in cell number and growth by increase in cell size or density). Exact differentiation of these types is difficult since bone growth in length, under normal conditions, is obviously accompanied by an increase in weight; and since it is probable that bone growth in weight is not necessarily productive of increments in length (2). The available data do not allow a complete separation of the two phases. From them, however, indications are obtained which are suggestive.

Records of skeletal size of the albino rat on age have been made by Jackson and Lowrey (3) and by Donaldson and Conrow (4). Their material was not treated from the dynamical point of view, nor was there a separation of the values according to sex. Such being the case, and because my study of the chemical differentiation of bone during growth showed that sex differences exist, the present analysis was undertaken, using the lengths and weights of the bones which served for the chemical investigation as the basic data (5, 6).

The material consisted of the humerus and femur of male and female albino rats 23, 30, 50, 65, 75, 100, and 150 days of age. The animals were healthy. They came from the Experimental Colony of The Wistar Institute, had a common inheritance, and were all raised under the same dietary and environmental conditions (described by Greenman and Duhring (7)). The number of bones available in

each age series and their distribution is given in the paper cited (5), as are the figures for the mean lengths and weights and their probable errors. The general method of preparation of the bones for measurement is described elsewhere (8). The strength of the macerating fluid and the time of maceration was adjusted to the age of the animal.

If the raw data be charted (charts not given) it is evident that bone growth in length and in weight exhibits two cycles between the ages of 23 and 150 days in the life of the albino rat. While these cycles, in all probability, are representable by curves based on the idea that the course of growth simulates that of an autocatalyzed monomolecular chemical reaction, as developed by Robertson (9), the paucity of intervening observations makes an attempt at formulation useless.

It has been found useful in the analysis of data of this nature to express the course of growth in terms of "growth capacity" (5, 6). This is a measure of the ability of unit measure of organ to add increments to itself in terms of initial quantities (1). In this case it represents "gm. per 100 gm. per day," and "mm. per 100 mm. per day" at the stated periods of observation. The growth capacity values are thus seen to be abstract values and hence directly comparable. A plotting of these values on an arithmetic grid instead of upon semilogarithmic paper makes possible a better visualization of the relations in the present instance, because of the cyclic character of the changes and the absolute magnitudes of the figures.

The results of the computations made from the raw data (5) are given in Chart 1.

Certain noteworthy systemic and sex relations in growth capacity in length and weight, and their consequences, are deducible from the curves.

In both sexes the growth capacity of the humerus in length is quantitatively more nearly like that of the femur than is growth capacity in weight. As a result the increase in difference in length between the two bones, which largely occurs during the period of active differentiation (23 to 65 days of age), is less in degree than the increase in weight difference, and the systemic difference in serially homologous bones in the adult as well as in the immature animal is less a difference in length than a difference in weight.

In both bones the growth capacity of the male in length is quanti-
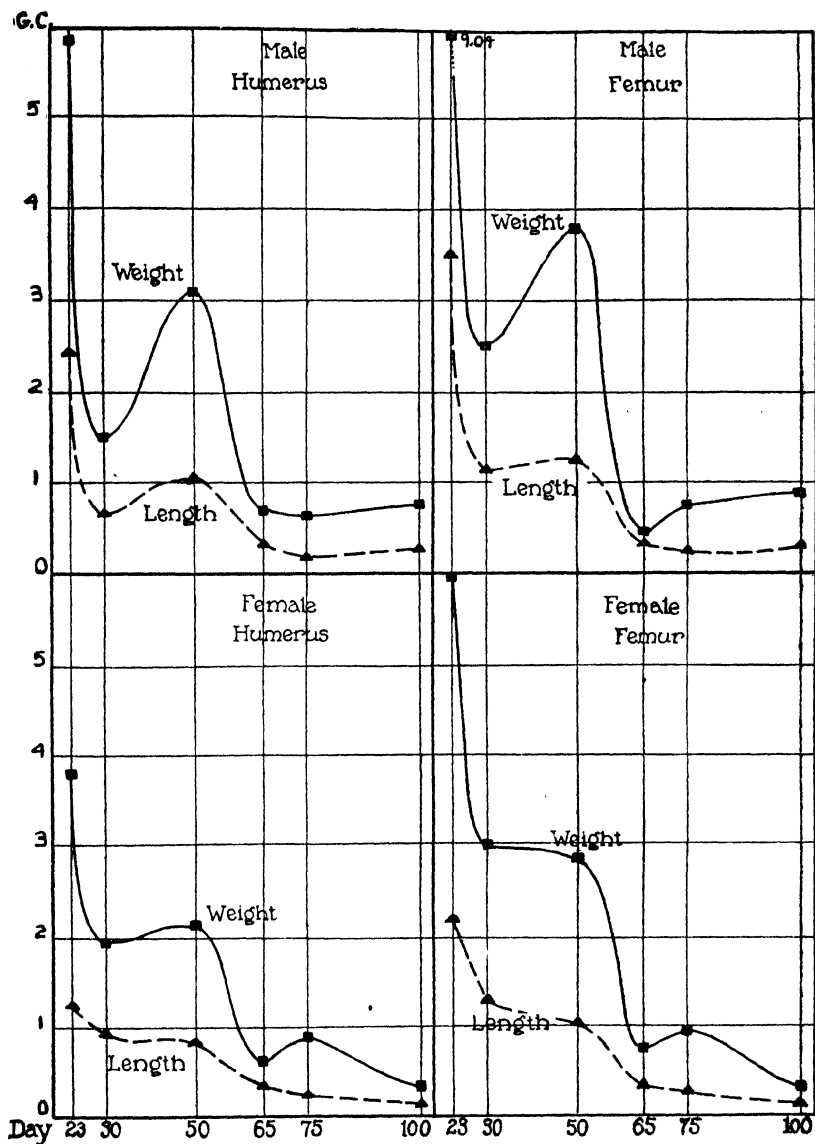
Chart 1.   The growth capacity in length and weight.

tatively more nearly like that of the female than is the growth capac-
ity in weight.   As a result, the sex difference in length increases to a

lesser degree than the sex difference in weight during the period of active differentiation, and the sex difference in isotopic bones in the adult as well as in the immature animals is less a difference in length than a difference in weight.

It thus appears that growth in length is less systemically and sex characteristic than growth in weight. When there is taken into account the known difference in mode of growth in weight by deposition of ash materials with the resultant systemic and sex differences in structure, and the mode of growth in length by cartilage increments (2), it is obvious that the phenomenon is consistent with the assumption that growth in length is largely a matter of increase in cell number, and growth in weight a matter of increase in cell size and density.

It is seen from the chart that the growth capacity in length for both bones of both sexes, at all ages, is less than the growth capacity in weight. As a result the length of the bones is only doubled while the weight is increased from 3 to 7 times according to the sex and structure. It is evident that the processes productive of bone strength are always more active than those concerned in longitudinal expansion or stature. Associated with this phenomenon is the fact that the body weight of the albino rat increases from 10 to 15 times its original value during the growth period from 23 to 150 days, while the body length is only a little more than doubled; and the fact that similar general relations between growth in stature and growth in weight exist in man (10).

This differential growth may be a particular expression of a general organic relationship between rate of growth by increase in cell number and rate of growth by increase in cell size. In addition it may be an expression of a response to a need for greater strength of supporting structure arising from the proportionately greater rate of growth in total body weight as compared with rate of growth in body length.

Turning now to a comparison of growth capacity in length and in weight on age, it is seen that marked retardations of both types of growth occur at 30 and at 65 days of age. These are attributable respectively to changes incident to weaning and puberty, as has been discussed in detail in an earlier paper (5). If the ratio

$$\frac{\text{Growth capacity in length}}{\text{Growth capacity in weight}}$$

be calculated and the values plotted as in Chart 2, it is at once evident that the retardation of growth capacity in length is definitely less in all cases than the retardation of growth capacity in weight.
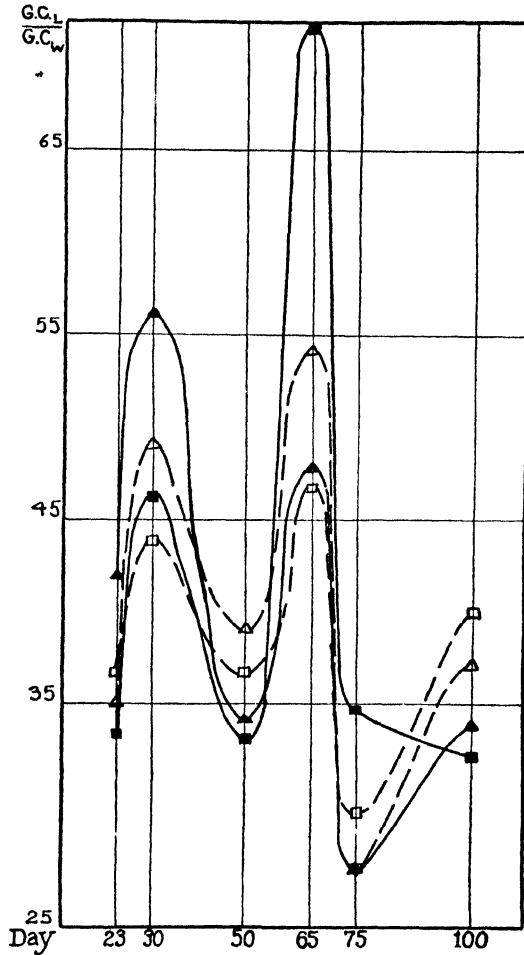
Chart 2. The ratio between the growth capacity in length and in weight.
Humerus: ♂ ▲———————▲ Femur: ♂ ■ ———————■
♀ △----------△° ♀ □-----------□

This greater stability of growth rate in length is also exhibited under conditions of potential stimulation such as exist at 50 days of

age. The phenomenon is best shown by a tabulation of the growth capacity at 50 days in terms of that at 30.

In all cases the extent of change in growth capacity induced by the change in physiological condition is less in length than in weight.

From this and the preceding fact, the conclusion is justified that bone growth in length is less susceptible to disturbance by factors arising at various stages of development than is bone growth in weight. The implication is that the processes concerned in growth by increase in stature (or cell number through cartilage increments (2)) are generally more resistant to the factors specifically associated with the physiological stage of development, than are the processes concerned in increase in strength of supporting structure (or growth by increase in cell size or density through ossification (5)).

TABLE I.

The Growth Capacity at 50 Days of Age in Terms of That at 30.

	Male.		Female.	
	Humerus.	Femur.	Humerus.	Femur.
Weight..................................	207.5	151.2	110.9	94.7
Length..................................	126.5	107.8	88.4	79.4

Notwithstanding this greater resistance of growth in length, it is a fact that the total percentage decrease in growth capacity on age for the period of observation (23 to 150 days) is practically the same for growth capacity in length as for growth capacity in weight. This is shown by plotting the terminal values on an arith-log grid as in Chart 3.

This fact indicates that in the long bones both types of growth are factored with equal intensity by age.

This is not the place to go into a differentiation between age and physiological stage of development as factors which must be separately considered when an analysis of growth is being made. A preliminary delimitation has already been given (11). The significant point, with regard to bone growth, is that while growth capacity in length differs from growth capacity in weight in the intensity of response to factors due to the physiological stage of development of the organism as a

whole, the response to the simple passage of time, or the age factor as such, is of equal degree in both types of growth, over the period represented by these observations. Whether this latter relation is a general relation or is confined to the humerus and femur is a matter which only future investigation can uncover.

It should be noted that at 65 days of age there occurs a stabilization of the growth capacity in length and weight, and an approximation
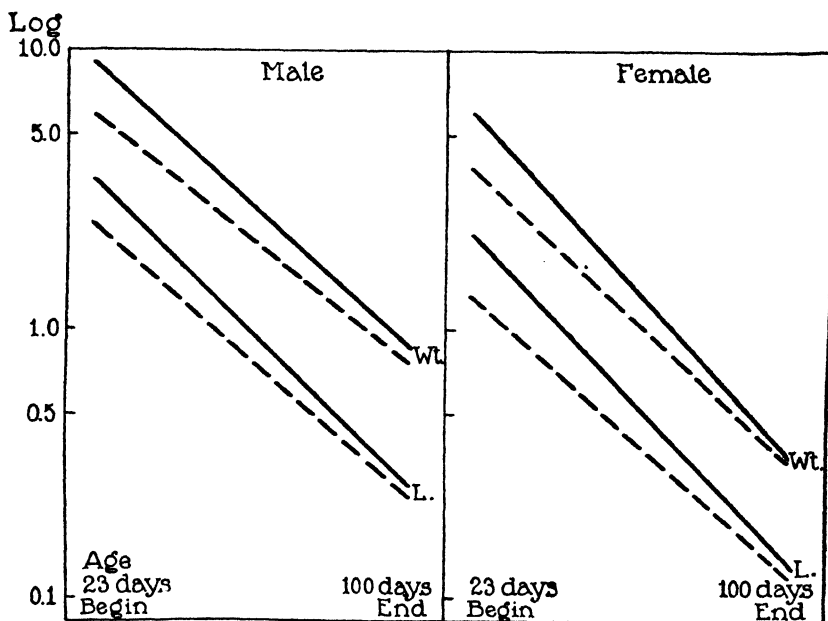
Chart 3. The decrease in growth capacity for the period of observation.
Femur ————
Humerus ----------

of the absolute values to a common level. This phenomenon has been discussed in an earlier paper (5). Attention is called to it here in order to show that the culmination of puberty has the same equilibrating effect on bone growth in length and weight as it does on the chemical differentiation.

If the relation between growth in bone length and growth in body length be compared with the relation between growth in bone weight and growth in body weight an interesting association is disclosed.

I have not given charts for the growth capacity values for body length and weight because the multiplicity of curves would make for confusion. It is enough to state that in both sexes the curve for growth capacity in body length is practically a replica of the curves for growth capacity in bone length, while the curve for growth capacity in body weight deviates markedly from those for bone weight during the period of active differential development.

Moreover, when the coefficients of correlation for bone length and body length, and for bone weight and body weight, are calculated (using all values regardless of age), it is found that in both bones of both sexes the correlation between the former is greater than between the latter pair of variables. The values are given in Table II.

TABLE II.

The Coefficients of Correlation between Bone Length and Body Length and Bone Weight and Body Weight.

	Male.			Female.		
	Bone length–body length.	Bone weight–body weight.	Difference. $\dfrac{r_l - r_w}{E_D}$	Bone length–body length.	Bone weight–body weight.	Difference. $\dfrac{r_l - r_w}{E_D}$
Humerus.	0.991±0.0012	0.971±0.0042	4.5	0.990±0.0013	0.982±0.0026	2.8
Femur....	0.992±0.0010	0.981±0.0027	3.8	0.990±0.0013	0.979±0.0030	3.3

These facts indicate that bone growth and body growth in length are more alike, both in kind and in degree, than are bone growth and body growth in weight. This relation is the expected since growth in bone length is largely skeletal growth while growth in body weight is only partly factored by growth of the osseous system. (The latter is but from 6 to 10 per cent of the total body weight within the age limits of these observations (4).) The high degree of correlation is noteworthy.

Linear regression is exhibited throughout in these relations, but since the regressions: *body length–body weight, bone length–bone weight,* etc., are non-linear, further analysis by the method of partial correlation is precluded.

Finally, the association between bone length and bone weight is

also high, but not so high as the correlation between bone size and body size. In the male the coefficient of correlation for the humerus is 0.938 ± 0.0087, and for the femur 0.947 ± 0.0074. In the female the value for the humerus is 0.938 ± 0.0087, and for the femur, 0.956 ± 0.0062.

SUMMARY AND CONCLUSION.

1. The growth capacity in length of the humerus and femur of male and female albino rats during the growth period from 23 to 150 days of age is less than the growth capacity in weight. This shows that the processes productive of bone strength are more active than those concerned in longitudinal expansion or stature.

2. Growth capacity in length is less affected than is growth capacity in weight by the systemic determinants and the factors incident to sex, weaning, and puberty. Since the findings as a whole are consistent with the assumption that bone growth in length is largely a matter of increase in cell number, while bone growth in weight is largely a matter of increase in cell size and density, the generalization is made that, in the bones at least, growth by increase in cell number is more stable than growth by increase in cell size and density.

3. A stabilization and approximation to a uniform level of growth capacity in both length and weight occurs at the culmination of puberty, which is quite like that taking place in the chemical differentiation at the same time.

4. Bone growth in length is more like body growth in length than bone growth in weight is like body growth in weight.

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ELECTROENDOSMOSIS THROUGH MAMMALIAN
, SEROUS MEMBRANES.

II. Comparison of Hydrogen Ion Reversal Points with Acetate and with Citrate-Phosphate Buffers.

By STUART MUDD.

(*From the Laboratories of The Rockefeller Institute for Medical Research.*)

Data comparing the hydrogen ion reversal points of human, cat, and dog serosæ with acetic acid-sodium acetate buffer and with citrate-phosphate buffer on the same experimental sites are presented in this communication. It is shown that the hydrogen ion reversal points are shifted a considerable fraction of a pH unit toward the acid side by the action of the polyvalent anions of the citrate buffer. The reversal points determined with the acetate buffer are believed to approximate natural conditions more closely, since the blood buffers contain polyvalent ions only in negligible amounts.[1] Furthermore, Michaelis and Rona[2] were unable to detect displacement of the pH of maximum flocculation and minimum cataphoresis of several proteins by the presence of Na, Cl, acetate, or phosphate ions, whereas the flocculation optimum of denatured albumin was shifted toward the acid side by citrate ion in the concentrations used by them by almost a full pH unit.[3]

The apparatus and experimental procedure were essentially as described previously.[4] Since the approximate reversal points were already known, it was possible to make a large number of runs with buffers whose pH values were close to those of the reversal points, thus securing accuracy.

[1] Van Slyke, D. D., Wu, H., and McLean, F. C., *J. Biol. Chem.*, 1923, lvi, 767.

[2] Michaelis, L., and Rona, P., *Biochem. Z.*, 1919, xciv, 225.

[3] Michaelis, L., and Rona, P., *Biochem. Z.*, 1910, xxvii, 48. Michaelis, L., and Davidsohn, H., *Biochem. Z.*, 1911, xxxiii, 456.

[4] Mudd, S., *J. Gen. Physiol.*, 1924–25, vii, 389.

For the acetate series acetic acid and sodium acetate stock solutions were mixed in such preparations as to give a graduated series of buffers, varying by 0.2 pH unit. The acetate mixtures were diluted to $M/50$. The citric acid-sodium phosphate mixtures were diluted to two twenty-fifths, the concentrations given by Clark,[5] that is, to about $M/85$ for the range used. The concentration of total citrate present in the range comprising the reversal points is given in Table I. All buffers were autoclaved to prevent growth of bacteria and moulds. Hydrogen ion concentrations were determined electrometrically.

The results of the experiments with both buffers are summarized in Table II. In any one row of the table only data derived from both buffers on the same sites are given, except where indicated in

TABLE I.

Undiluted buffer. pH	Diluted buffer. Mean pH.	Total citrate concentration. Gm. ions per liter.
4.0	4.27	.00492
4.2	4.46	.00469
4.4	4.66	.00447
4.6	4.91	.00426
4.8	5.14	.00406
5.0	5.49	.00388

Column 1 (dog peritoneum, cat and dog pleura). The reversal points were determined first with acetate, then with citrate buffer, without change of experimental site. The values published in the preceding paper[4] are given in parentheses under the several categories.

The hydrogen ion reversal points determined with the phosphate-citrate buffer are consistently lower (more acid) than those found with the acetate mixtures, as is shown in the last column of Table II. The amount of this shift in reversal point for the lean pericardial experimental sites is about one-half a pH unit; somewhat less for pleura and mesentery. For fat pericardial sites and for the human mesenteries which, unlike the animal mesenteries, contain a thick fat deposit, the shift is about one-quarter of a pH unit.

[5] Clark, W. M., The determination of hydrogen ions, Baltimore, 2nd edition, 1922, 116.

TABLE II.

No. of experiments.	Species.	Membrane.	State of animal.	Condition of membrane.	Mean reversal point, acetate buffer. pH	Range of variation, acetate. pH	Mean reversal point, citrate buffer. pH	Range of variation, citrate. pH	R. P. acetate-R. P. citrate buffer. pH
4	Human.	Peritoneum.	Post mortem.	Fat.	5.02 ± 0.031	.19	4.81 ± 0.024	.15	.20
4 (7)	Cat. "	"	Living. "	Lean.	4.86 ± 0.028 —	.20	(4.36 ± 0.029)	(0.3) —	(.50)
4 (4)	" "	" "	Dead. "	" "	4.91 ± 0.023 —	.12	(4.80 ± 0.050)	(0.3) —	(.11)
5 acetate. 2 both. (9)	Dog. "	" "	Living. "	" "	4.96 ± 0.018 —	.16	4.635 (4.49 ± 0.064)	.11 (0.9)	.32 (.47)
4 acetate. 2 both. (2) 1 (1)	" " " "	" " "	Dead. " " "	" " Fat. "	4.95 ± 0.040 — 5.03 —	.27	4.69 (4.62) (5.0) —	.22 (.15) —	.26 (.33) (.03)
4 1	Human. "	Pericardium. Pericardium and pleura fused.	Post mortem. "		5.53 ± 0.043 5.72	.29 —	5.12 ± 0.074 5.06	.48 —	.41 .66
5 (2) 5 (2)	Cat. " " "	Pericardium. " "	Dead. " "	Lean. Fat. "	5.58 ± 0.063 — 5.11 ± 0.047 —	.47 — .41 —	5.07 ± 0.052 (4.9) 4.85 ± 0.080 (5.07)	.46 (0.6) .69 (.25)	.51 .68 .26 (.04)
4 (8) 4 (6)	Dog. " "	" " "	Lean. " Fat. "	5.87 ± 0.033 — 5.34 ± 0.131 —	.21 — .90 —	5.31 ± 0.003 (5.26 ± 0.032) 5.09 ± 0.074 (5.08 ± 0.021)	.02 (.45) .48 (0.2)	.56 .61 .25 (.26)
5 acetate. 1 both. (5)	Cat. "	Pleura. "	" "	Lean. "	5.09 ± 0.069 —	.63 —	4.82 (4.32 ± 0.058)	— (0.5)	.27 (.77)
4 acetate. 3 both. (4) 1	Dog. " "	" " "	" " "	" " Fat.	5.18 ± 0.038 — 4.93	.23 — —	4.78 (4.97 ± 0.019) 4.80	.12 (0.1) —	.40 (.21) .13

The mean hydrogen ion reversal points for fat pericardia are in this series considerably lower than those for lean pericardial sections of the same animals. The difference between lean and fat values is .50 pH unit with the acetate, and .22 pH with the citrate buffer. The reversal point values for fat pericardia in this series are, furthermore, considerably more variable than those for the lean pericardia, as may be seen from the relatively large range and probable error values for the fat membranes. Finally it has been noted in several experiments of this series that the apparent reversal point has shifted toward the acid side during the perfusion of many hours, or even several days necessary to reach a stable reversal point with the fat membranes. All of these facts can be explained by the very reasonable assumptions that the thin protoplasmic shells of the fat cells have been injured to greater or less degree by the prolonged perfusion, thus exposing to the perfusing buffer electronegative fat with little or no affinity for the citrate ions. The assumption of injury seems to be borne out by examination of histological sections of the experimental sites which have usually shown channels several cell diameters wide, bordered by damaged fat cells.

Hydrogen ion reversal points characteristic for each of the three serous membranes of the several species were found with the acetate buffer, as had been the case with the phosphate-citrate mixtures. Inspection of Column 6 of Table II will show values for peritoneum, pleura, and pericardium, differing by amounts many times the probable errors of the mean, the precision measure given. The reversal point values for each of the membranes of the dog are again appreciably higher (more alkaline) than for the corresponding membranes of the cat. Differences in reversal point were thus found again between different species and between the several tissues of each species. The human pericardial values, pH 5.53 ± 0.043 for acetate and 5.12 ± 0.074 for citrate buffer are close to lean pericardial reversal points for the cat. The human peritoneal values, 5.02 ± 0.031 for acetate and 4.81 ± 0.024 for citrate, are hardly comparable with the animal values because of the deposit of fat in the human mesentery.

The ready reversibility of the charge of the membranes, even of mesenteries of living animals, has again been repeatedly demonstrated. Fig. 1 gives the data from an experiment with a single site of the mesen-
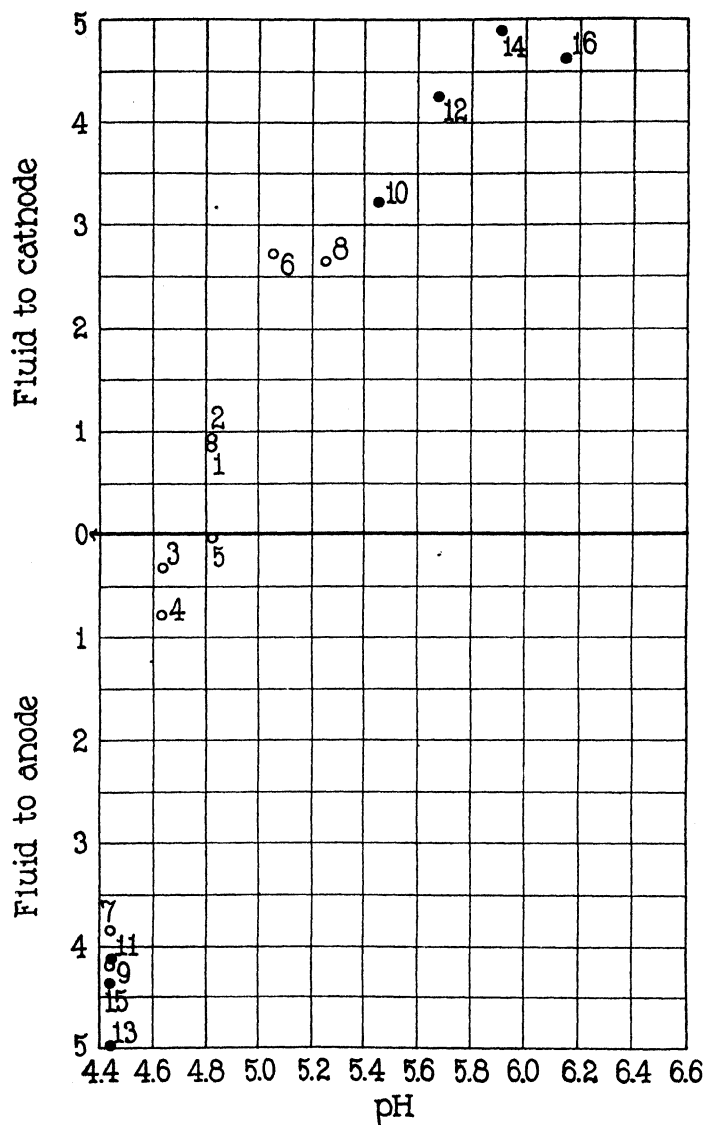
FIG. 1. Mesentery of living cat. Acetate buffer. Abscissæ indicate pH of buffers bathing outside of membrane. Ordinates represent volume of liquid transported in c.mm. per minute per milliampere of current. Ordinate values for open circles approximate only. Runs numbered in the order made. Chart illustrates ready reversibility of charge on membrane.

tery of a living cat.   The order of the runs is indicated by the numbers on the diagram.   The sign of the electrokinetic P.D. of this area of mesentery was changed some twelve times during the course of $2\frac{1}{2}$ hours.   It is somewhat idle to speculate whether or not the actual mesenteric section used was living throughout the procedure, but at least little gross change was observable; the experimental site, when removed from the electrode vessel, could scarcely be distinguished from the neighboring mesentery.

Little or no difference has been found in the present series between the reversal points of the mesenteries of living animals and of similar or identical sites within the first hour or two after the death of the animals by bleeding.   It was suggested in the preceding paper[4] that the low apparent reversal points of the mesenteries of living animals there reported were referable to admixture of the perfusing buffer with the blood buffers in the membrane.   A much more thorough washing with the perfusing buffer was given the experimental sites of the present series before making the runs, and this mixture with buffer salts in the membrane seems to have been largely eliminated thereby.

The reversal points of the pleuræ of the present series were determined from 2 to 24 hours after bleeding the animals to death (except for the fat dog pleura which was 90 odd hours post mortem), and the reversal points of the pericardia from 1 to 8 days post mortem.   The membranes were preserved in a moist chamber in the ice box.   No postmortem shift in reversal points during these intervals was detected.

A discrepancy may be noted between the reversal point for cat pleura previously recorded and the values of the present series.   The present values are believed to be more nearly correct.   The low values given previously are interpreted as probably due to insufficient washing with the perfusing buffer of cat pleural sections, which in several instances were thickened by containing the phrenic nerve.

Reversal points of normal and pathological human serosæ have been determined in the present series without any correlation being detected.   Since the great bulk of these membranes, whether normal or pathological is white fibrous connective tissue and fat cells, the

effect of pathological infiltration or fibrin deposition upon electro-kinetic behavior would be expected to be slight.

Northrop and Kunitz have demonstrated complex ion forma-tion between isoelectric protein and Zn^{++}[6] and between protein and other ions.[7] du Noüy, on the other hand, has given evidence of ad-sorption of salt by surface films of serum proteins.[8] The data at hand do not seem to afford a basis for discriminating between the possible mechanisms which might bring about the shift in hydrogen ion reversal point with citrate buffer here recorded.

SUMMARY.

The hydrogen ion reversal points of human, dog, and cat serous membranes have been determined with acetic acid-sodium acetate buffer mixtures, and are compared with the reversal points of the same membranes estimated with citrate-phosphate mixtures. The values with acetate buffer are about one-quarter of a pH unit higher (more alkaline) for fat membranes and almost one-half a pH unit higher for lean membranes. The acetate values are believed to correspond more closely to the true hydrogen ion reversal points.

The reversal points are again found to be characteristic for mem-brane and species. No evidence of a postmortem shift in reversal point has been found. The charge of the membranes even in the liv-ing animal is capable of ready and repeated reversal.

[6] Northrop, J. H., and Kunitz, M., *J. Gen. Physiol.*, 1924–25, vii, 25.

[7] Personal communication from the authors.

[8] du Noüy, P. L., *J. Exp. Med.*, 1922, xxxv, 733.

THE INFLUENCE OF THE INTENSITY OF LIGHT ON THE RATE OF GROWTH AND DURATION OF LIFE OF DROSOPHILA.

BY JOHN H. NORTHROP.

(From the Laboratories of The Rockefeller Institute for Medical Research.)

Although light is one of the most striking attributes of the environment of living organisms, the results of practically all of the experiments designed to show the effect of light on the normal growth processes of animals have been negative. This is the more surprising in view of the marked effects which have been reported in connection with abnormal or pathological conditions. Aseptic cultures of *Drosophila* have been propagated in this laboratory for over 200 generations and during that time have been kept in the dark except for occasional exposure to diffuse daylight, and they would seem to be favorable material for a study of the influence of light on normal growth. The present experiments were undertaken to determine whether or not the rate of growth and total duration of life of these insects would be affected by variations in the intensity of light.

EXPERIMENTAL PROCEDURE.

Source of Light.—The light source was a concentrated filament Mazda bulb of 150, 500, or 1,000 watts. The bulb was immersed in a vessel of running water and surrounded by a layer of about 1 cm. water. The intensity was measured approximately by a Lummer-Brodhun contrast photometer against a standard Hefner amyl acetate lamp. The bulbs were frequently changed.

Culture Medium—The flies were grown and transferred in 500 cc. Pyrex flasks having a side arm as previously described.[1] For the rate of growth of the larvæ 10 cc. of a suspension of 40 gm. of yeast in 100 cc. of 2 per cent glucose agar were placed in a 500 cc. flask and sterilized. A number of freshly hatched flies from the stock culture were then put in the flask and the flask placed in the dark. After 24 hours the flies were removed and the flask placed in the

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[1] Northrop, J. H., *J. Gen. Physiol.*, 1919–20, ii, 317.

proper position. The imagos were raised on yeast in the dark, and were then transferred to glucose agar alone, which is sufficient for their maintenance but insufficient for growth of the larvæ so that it was unnecessary to transfer the adult flies. About 100 larvæ or imagos were used in each experiment, and the probable error of the mean was in all cases between 2 and 3 per cent.

The experiments were made in a constant temperature room in which the air was circulated with a powerful fan. Temperature reading with a mercury thermometer at different distances from the light showed at times an increase of about 1°C. at a distance of a foot from the light and it is possible that this is in part the cause of the increased growth rate at this distance.
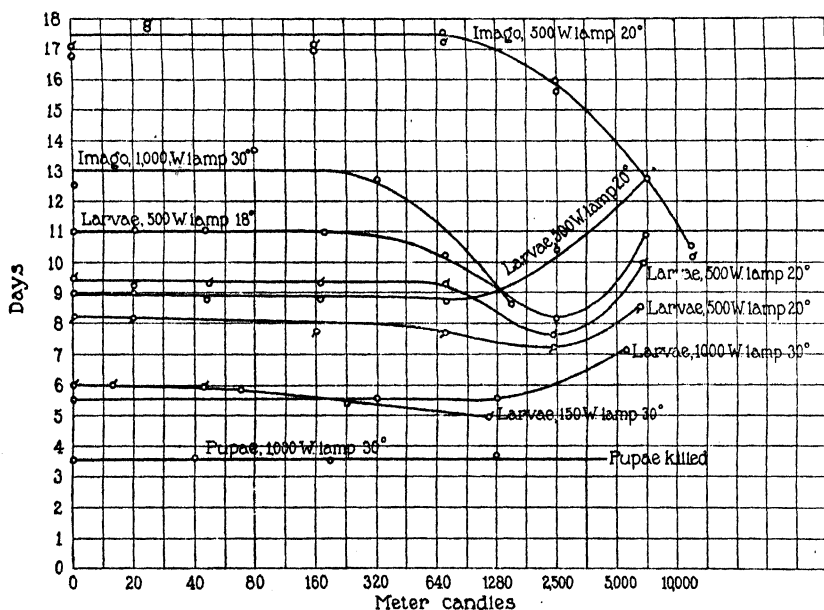
FIG. 1. Influence of the intensity of light on the rate of growth and duration of life of *Drosophila*.

The results of the experiments are shown in Fig. 1, in which the duration of the various stages in days has been plotted against the logarithms of the intensity of illumination expressed in meter candles. This intensity was varied by varying either the distance from the light, assuming the inverse square law, or the intensity of the light itself. The results show that up to light intensities of about 600 meter candles there is no change either in the rate of growth or in the duration of

life of the imago. Above this intensity the duration of life of the imago rapidly decreases while the duration of the larval period decreases slightly and then increases. There is no effect on the pupæ except that above 5,000 meter candles the pupæ are killed. As is the case with temperature the pupal stage is the most sensitive. It is possible, as stated above, that the decrease in the larval period (increase in the rate of growth) is due in part to a temperature difference. The result is in any case similar to that obtained with temperature in which it was found that there was an optimum temperature for the larval growth but not for the duration of the imago stage.[2] Apparently injury from either temperature or light results at first in an increase in length of the larval or growth period but a decrease in the imago or adult period, whereas in the normal range both periods are influenced in the same direction. It is interesting to note in this connection that anything which increases the rate of growth, *i.e.* shortens the growth period, is usually considered a beneficial effect, whereas anything which increases the rate of life, *i.e.* shortens the adult period, is considered harmful. To be consistent it would seem that a decrease in the rate of growth (which is equivalent to prolonging the length of the growth period) is just as beneficial to the organism as increasing the duration of the adult period, and the writer has shown in fact that decreasing the rate of growth results in adding an equivalent number of days to the total duration of life.[3]

The Relation between the Duration of Life and the Intensity of Illumination.

The experiments show that the duration of the imago stage is not affected within the experimental error up to 640 meter candles and that above this point the duration of life decreases rapidly and approximately in proportion to the logarithm of the intensity.

There is probably no doubt that the observed effect is the sum of two reactions, first, the normal "aging," and second, the effect of the light. The total rate of reaction, K_o, would therefore be the sum of two reaction velocities, one the normal velocity, K_n, and the other the velocity K_l due to the light; *i.e.*, $K_o = K_n + K_l$.

[2] Loeb, J., and Northrop, J. H., *J. Biol. Chem.*, 1917, xxxii, 103.
[3] Northrop, J. H., *J. Biol. Chem.*, 1917, xxxii, 123.

When the illumination is 0, $K_o = K_n$ so that the value of this velocity can be determined from the duration of life in the dark. It is necessary to know also in what way the rate of reaction due to the light varies with the intensity of illumination. It might be expected that the rate of reaction would increase with the logarithm of the intensity as predicted by the Weber-Fechner law. This assumption would predict, however, that the observed rate (reciprocal of the time) would give a straight line if plotted against the logarithm of the illumination. This is evidently not the case, however, unless the entire first part of the curve up to 640 meter candles be neglected. It is true that from that point an approximately straight line is obtained. It is known, however, that in most photochemical reactions the velocity of the reaction is *directly* proportional to the intensity of illumination. Using this assumption, the velocity of the reaction due to the light would be proportional to the light intensity; *i.e.*, $K_e = CI$. The equation for the whole process, assuming the velocity equal to the reciprocal of the time, therefore would become

$$K_o = \frac{1}{T_o} = K_n + CI \qquad (1)$$

As stated above, when I is zero, $K_n = \frac{1}{T} = 5.7$. If this value is substituted at the point where $I = 2,500$, C is found equal to 27 $\times 10^{-5}$. If the assumptions used are justified, it should be possible to calculate the experimental results from these values of K and C. That this is true is shown in Table I. The observed values for the duration of life at varying intensities agree closely with those calculated by means of equation (1).

According to the above mechanism the light does not simply speed up the normal "rate of aging" but produces a separate reaction or reactions. This can be tested by noting the effect of short exposures. Table II contains the results of an experiment in which the flies were exposed for 3 days to 10,000 meter candles. This experiment was carried out with a different culture of flies and was at 25°C. so that the results are not directly comparable with the preceding ones. The flies which were not exposed to the light lived on the average 13.5 days, those that were continuously exposed lived 3.9 days, and those

that were exposed for 3 days and then placed in the dark lived 7.9 days. Very few of them had died before removal from the light. If the light merely accelerates the velocity of the normal reaction, the flies after 3 days exposure should be the same as those that had lived the same percentage of their life in the dark, that is, the equivalent age of these flies compared to a culture kept always dark should be

TABLE I.

Duration of Life at Various Intensities Calculated from

$$T = \frac{1}{5.7 + 2.8 \times 10^{-4}I}$$

I	Duration of life.	
	Observed.	Calculated.
meter candles	*days*	*days*
0	17.5	(17.5)
160	17.5	17.4
640	17.5	17.0
1,280	17.0	16.5
2,500	15.7	(15.7)
5,000	14.0	14.0
10,000	11.3	11.6

TABLE II.

Days at 10,000 meter candles..............	–	3	30
Days in dark...........................	30	30	–
Average total duration of life.............	13.0	8.0	3.8
	14.0	7.8	4.0
	13.5	7.9	3.9

Equivalent age $= \dfrac{3}{3.9} \times 13.5 = 10.6$

$\dfrac{3}{3.9} \times 13.5 = 10.6$ days, and they should therefore live about 3 days after placing in the dark. Actually, they lived nearly 5 days. The experiment shows, therefore, that the light produces an effect other than the mere acceleration of the normal "aging rate" and that this effect is not completely reversible, since in case of complete reversibil-

ity or recovery the short exposure would have had no effect on the total duration of life.

It was noted previously that the upper temperature limit for continued growth of these cultures, 27°C., is surprisingly low[1] and it seemed possible that this might be due to the fact that the experiments were made in the dark. It was found, however, in experiments in which the cultures were exposed to diffuse daylight, through glass, that the upper temperature limit remained the same.

SUMMARY.

The duration of the larval and imago periods of *Drosophila* cultures which had been previously grown in the dark for 200 generations has been determined at various light intensities.

1. The duration of the larval period is shortened slightly at intensities around 2,500 meter candles, but becomes increasingly longer at higher intensities. The larvæ are killed by continuous exposure to light of 7,000 to 10,000 meter candles.

2. The pupæ are killed at intensities greater than 5,000 meter candles.

3. Above 1,000 meter candles the duration of the imago period is rapidly shortened.

4. The duration of life of the imago at different intensities of illumination can be quite accurately predicted by assuming that the light produces an independent "rate of aging" which is proportional to the intensity of the light.

5. The result of short exposure of the imago shows that light does not merely accelerate the normal "rate of aging," and also that the effect is only partially reversible.

Diffuse daylight does not affect the upper temperature limit of growth.

THE INACTIVATION OF TRYPSIN BY X-RAYS.

By HARRY CLARK and JOHN H. NORTHROP.

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The inactivation of trypsin by the rays from the active deposit of radium emanation has been studied by Hussey and Thompson.[1] They find that in dilute solution trypsin is inactivated at a rate which is proportional to the intensity of the radiation and to the concentration of the solution; that for constant intensity the reaction would follow the simple exponential law which indicates a monomolecular reaction. In concentrated solution, it is not inactivated. They state also that a 2 hour exposure to hard, filtered x-rays produces no effect although soft, unfiltered rays produce considerable inactivation.

Although physical theory indicates that radiation produces changes in matter through the medium of ionization, it has not been shown conclusively that various apparently characteristic effects, produced by various kinds of rays, result from corresponding characteristic intensities and distributions of ionization. For this reason it has seemed desirable to study the inactivation of trypsin under soft x-ray.

The experiments to be described were made on solutions of Fairchild's trypsin powder. These solutions, of various degrees of trypsin concentration, were made up in .05 M, pH 5.4 acetate buffer. The results are in good accord with those of Hussey and Thompson.

Method and Apparatus.

The x-ray outfit has been described.[2] A broad-focus Coolidge tube is operated on 60 cycle current rectified by kenetrons; it is enclosed in a lead-lined cabinet and kept cool by a fan. This outfit is capable of continuous operation without a variation of voltage or current exceed-

[1] Hussey, R. G., and Thompson, W. R., *J. Gen. Physiol.*, 1922–23, v, 647.
[2] Clark, H., *Am. J. Roentgenol. and Radium Therap.*, 1924, xi, 445.

ing 2 per cent. In this work it was run at 30 kv. (peak) and 22 milliamperes, the mean target distance being 14.7 cm.

It proved rather difficult to provide conditions suitable for exposing the trypsin. Even with the solution close to the tube, exposures of several hours duration were required to produce any considerable effect; consequently the spontaneous inactivation was also considerable, even at a temperature of about 0°C. To keep the solution at this temperature necessitated efficient cooling apparatus and careful shielding·from the heat of the tube.

A strip of sheet lead was set into a shallow round tray of enamelled iron, 10 cm. in diameter, so as to divide it into two equal compartments, after which the tray was dipped in hot paraffine to coat the surface and to make the compartments water-tight. The control solution was put in one side and the solution to be radiated in the other. A sheet of lead was placed over the control to shield it from the x-ray, and to ensure perfect shielding it was allowed to overlap the radiated solution slightly, thus casting on it a shadow about 5 sq. cm. in area. The cross-section of one-half of the tray being 39.2 sq. cm., only 87 per cent of the radiated solution was actually exposed. This factor will have to be considered later.

The tray was supported in a small water bath through which a strong current of ice water was maintained by means of a motor-driven gear pump. To shield the solutions from the heat of the tube and to prevent evaporation, a metal frame, carrying four sheets of thin paper parallel and about .5 cm. apart, was so placed over the tray as to close it to air currents. The fan which served to cool the tube maintained a current of air between the sheets of paper. With this arrangement the solutions were kept continuously at a temperature of about .5°C. 15 cc. of solution were used in each run, the depth being .382 cm.[3]

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[3] In the first experiments, two of which are here used, the whole tray was used for the solution to be radiated, the control being kept on ice elsewhere. The conditions of radiation were, therefore, different from those described; the depth was slightly less, the distribution of radiation slightly different, and the difference in temperature between the two solutions may have been as great as .5°C. These runs are certainly comparable with the others to a good degree of approximation. No calculations are based on them, however; they are used in this paper only to show the general magnitude of the effect.

At proper times during each run, the solutions were stirred and 1.5 cc. samples were taken. Whenever a sample of radiated solution was taken, an equal volume of control solution was added to keep the depth constant. This process was compensated by calculation; since few samples were taken, the correction was negligible in some cases and small in the others. The trypsin content of the samples was measured by Northrop and Hussey's viscosity method.[4] This method involves sufficient dilution to insure that all of the enzyme exists in the active state. The determinations are independent, therefore, of the state in which it existed in the original sample.

RESULTS.

The results of five runs are shown in Fig. 1 in which logarithms of trypsin content per cc., in arbitrary units, are plotted against the length of exposure in hours. The circles refer to the control solution (spontaneous decay) and the dots to the exposed solution (x-ray plus spontaneous decay). Each point is the mean of two determinations. Nos. 1, 2, and 4 were ordinary solutions. No. 3 was almost wholly inactivated by heat before use, and No. 5 was dialyzed before use. Nos. 2 and 3 are the ones referred to in Foot-note 3.

The percentage concentrations shown in figures on the chart for the various solutions refer to the dry weight of trypsin powder used in making them. The dry material contains an unknown and somewhat variable amount of foreign material, and the enzyme itself suffers spontaneous inactivation both in the dry state and in solution; in consequence, the figures given do not correspond accurately to the initial values plotted.

Quantitative work with enzymes entails usually an uncertainty of several per cent. With the exception of a few points, the various straight lines represent the courses of the various runs within the expected limits of error. Although straight lines may not represent the law of inactivation accurately, they furnish the simplest basis for discussion.

[4] Northrop, J. H., and Hussey, R. G., *J. Gen. Physiol.*, 1922–23, v, 353.
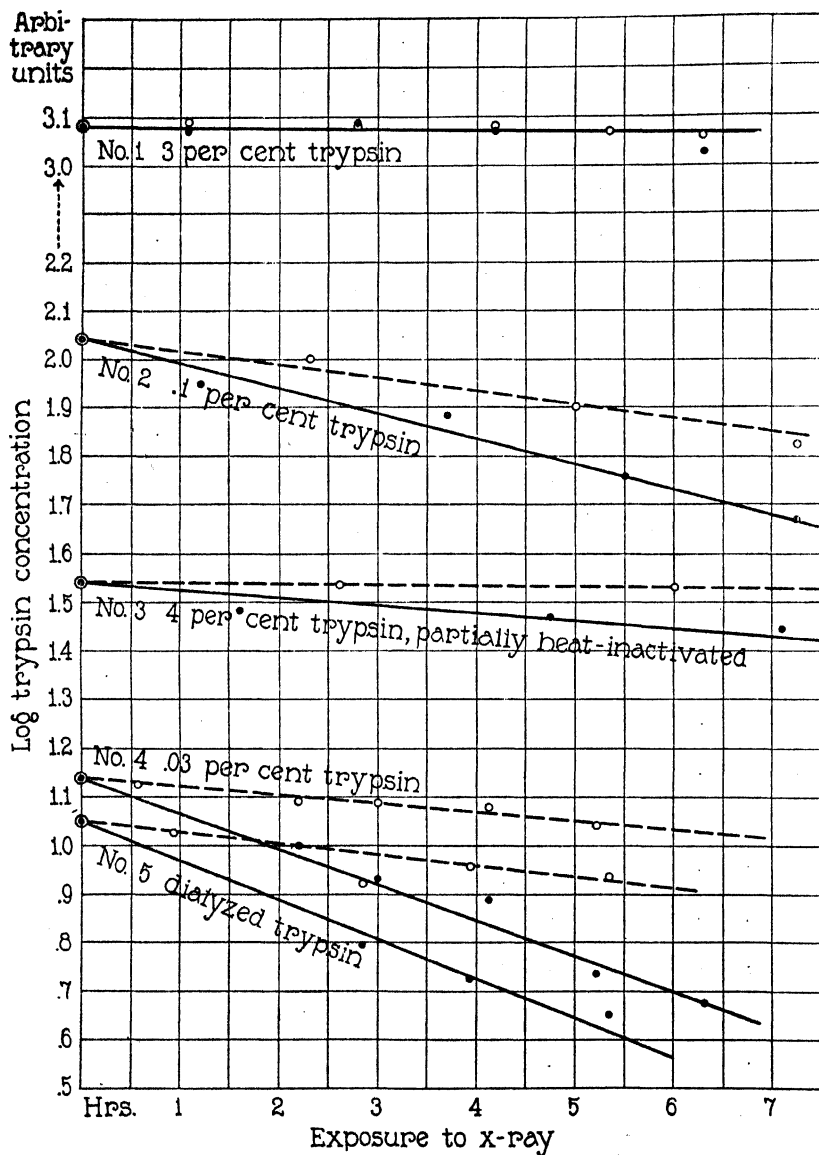
FIG. 1. Inactivation of trypsin solutions by x-rays.

DISCUSSION.

Theory of Enzyme Solutions.—Northrop has shown[5] that, in general, in solutions of trypsin containing foreign material of certain sorts the trypsin exists partly in the combined or inactive state, and partly in the free or active state. The reaction is reversible and obeys the law of mass action. Therefore, the point of equilibrium depends both on the concentration of trypsin and on that of the foreign matter. In concentrated solution most of the enzyme is combined; in dilute solution it is mostly active. The rate of spontaneous inactivation of combined trypsin is negligible compared to that of free trypsin.

X-Ray Inactivation and the State of the Enzyme.—The chart shows that the inactivation by x-ray and the spontaneous inactivation run parallel within reasonable limits; both are slight in Nos. 1 and 3, and great in Nos. 4 and 5. No. 2 lies between the extremes. If No. 3 be left out of account, the effects are seen to increase with the dilution over the range studied. All of the solutions were made from the same stock of trypsin powder. With the exception of Nos. 3 and 5, they represent, therefore, about the same ratio of enzyme to foreign matter. The ratio of free to combined trypsin increases consequently with the dilution. Before use, No. 3 was almost entirely inactivated by heat, which presumably did not alter the amount or the condition of the impurities to any great extent. Although trypsin is present in this solution in very small amount, it exists almost entirely in the combined state; which accounts for the stability under both x-ray and heat. Solution 5 was dialyzed several times before use to remove as much of the impurity as possible. The chart discloses nothing peculiar about the behavior of this solution; apparently the amount of impurity in the stock was not sufficient to hold any appreciable part of the trypsin in combination at very low concentration, so dialysis was unnecessary. On the basis of all of these experiments, and of Northrop's theory,[5] we conclude that x-ray affects predominantly free trypsin.

On the more concentrated solutions, the effects are too small to throw any light on the law of inactivation. Straight lines fit Experiments 4 and 5 fairly well, though the latter is represented by too few

[5] Northrop, J. H., *J. Gen. Physiol.*, 1921–22, iv, 261.

points to be of much value. The exponential law and a monomolecular law are indicated. Both of these conclusions are in accord with those of Hussey and Thompson.[1]

Ionization Theory.—It is quite generally accepted on the basis of physical theory that x-rays effect primary changes in matter only through the ionization produced by the emission of electrons with high speed; *i.e.*, beta rays. Gamma rays are, of course, merely hard x-rays. On this view, x-rays of all wave-lengths and the beta and gamma rays of radioactive substances should produce the same changes if their intensities were so adjusted as to produce equal rates of ionization per unit volume.

The ionization of liquids and solids by radiation cannot be measured directly, but it has been shown[6] that substances composed only of light elements, water, air, organic substances, in whatever state, are ionized in proportion to their density. From measurements made in air we can, therefore, estimate the ionization of the enzyme solution.

Hussey and Thompson used radium emanation enclosed in glass tubes thick enough to stop all of the alpha particles. That they were not so thick as to interfere appreciably with the passage of beta particles is indicated by the fact that several different tubes gave the same results. The slight interference may be considered as cancelling the almost negligible effect of the gamma rays. The trypsin solution surrounded the tube in a layer sufficiently thick to stop practically all of the beta rays. As a first approximation then it may be assumed that the ionization of the solution resulted from total absorption of the beta rays from Radium B and C.

Moseley and Robinson[7] state that the beta rays from B and C in equilibrium with 1 gm. of radium produce 9×10^{14} pairs of ions per second in air, if completely absorbed. To reduce the time unit from seconds to hours, and the curies to millicuries, we multiply this number by 3,600 and divide by 1,000. Thus, one millicurie-hour represents 3.24×10^{15} pairs of ions. 2.95 cc. of solution were used; the number of pairs of ions per cc. per millicurie-hour is, therefore, 1.10×10^{15}. From the curves given by Hussey and Thompson, it may be seen that 240 millicurie-hours reduced the activity of the trypsin

[6] Crowther, J. A., *Proc. Roy. Soc. London, Series A*, 1907–08, lxxx, 186.
[7] Rutherford, E., Radioactive substances and their radiations, 1913, 230.

to half value ($\log_{10} 2 = .301$). Multiplying $1.10 \times 10^{15} \times 240$, we find the corresponding number of pairs of ions per cc. to be 2.63×10^{17}.

Leaving this result for the moment, let us estimate the total ionization per cc. required to reduce the trypsin to half value by the x-rays in our experiments. The rate of ionization in air was measured by apparatus previously described.[2] At a target distance of 27 cm. but with all other conditions as in the work with the enzyme, 5.42×10^{12} pairs of ions were produced per second per gm. of air. The possible influence of the metal tray on the enzyme was investigated by taking readings with and without the empty tray directly under the ionization chamber and no measurable effect was found. The effect of scattering, with these very soft rays and under the conditions of the work, is negligible. The proof of this is given in the report of certain other experiments[8] where the conditions of measurement were the same as in the present case except that a different tube was used and also a filter of thin bristol board practically identical in stopping power with the four sheets of paper used in the present work. In fact the actual ionization value used in that work differs from the one here used by only about 10 per cent, that being the difference in efficiency between the old tube and the new one.

The ionization per cc. of enzyme solution per hour may be taken as equal to that in a gm. of air in the same time and under the same circumstances. To estimate it, we must interpolate the value at 27 cm. to the actual target distance, 14.7 cm., by the inverse square law and multiply by two correction factors. The first of these factors, .87, has already been computed; it accounts for the shadow cast on the radiated solution by the lead screen. The second factor, .89, takes account of the falling off of the ionizing power of the rays in traversing the solution. This was studied with the ionization chamber and a sheet of wax of the same weight per cm.[2] as the solution. The absorption by the wax amounted to 22 per cent, and the factor, .89, represents, therefore, the average relative intensity throughout the solution. Since the chamber was stationary, the use of this factor is consistent with the use also of the mean target distance. Multiplying 5.42×10^{12} by the square of $(27 \div 14.7)$, by .87, by .89, and by 3,600 (seconds to hours), we get 5.09×10^{16} for the number of pairs of ions produced

[8] Clark, H., and Sturm, E., *J. Exp. Med.*, 1924, xl, 517.

per hour in 1 cc. of solution.   Experiment 4 (see Fig. 1) represents the concentration nearest that used by Hussey and Thompson (.0375 per cent).   The difference between the ordinates of the two curves of Experiment 4 is .301 or $\log_{10} 2$ at $5\frac{1}{2}$ hours, which is, therefore, the time required for half-inactivation by x-ray alone.   This corresponds to $5\frac{1}{2} \times 5.09 \times 10^{16}$ or 2.80×10^{17} pairs of ions per cc.

The ratio of this value to the one estimated above for the work with radium emanation is 1.065.   The agreement is better than could have been expected.   If not fortuitous, it substantiates the hypothesis already stated, that radiations of various sorts do not produce characteristic effects—only ionization.   It indicates also that the trypsin in the commercial preparation is a single substance rather than a mixture of various substances; otherwise the two different stocks considered in the estimate could scarcely have given comparable results.

Nature of the Process of Inactivation.—Under ionization of random distribution, the probability, P, that at least one free electron will appear in (or disappear from) a certain small portion of the solution, within the time interval dt, is given by

$$P = n \, v \, dt.$$

where n is the number of electrons (*i.e.* the number of pairs of ions) set free in 1 cc. of solution in 1 second; and v is the volume of the portion considered.   If n is large, and if we consider a large number, N, of portions, all of the same volume, v, then P becomes the actual fraction of them in which at least one free electron appears in time dt; that is,

$$P = - \frac{dN}{N} = n \, v \, dt,$$

$$- \log_e N = n \, v \, dt + k,$$

and, since $N = N_0$, when $t = 0$,

$$- \log_e N_0 = k,$$

and

$$\frac{\log_e N_0}{N} = n \, v \, t.$$

This is the simple exponential law previously referred to.   No theory of multiple or successive ionizations would yield this simple relation. For "inactivation" to half-value $\frac{N_0}{N} = 2$ and $v = \frac{.692}{nt}$.

Setting nt equal to 2.80×10^{17}, the number of pairs of ions per cc. corresponding to half-inactivation of free trypsin as found in our experiments, we find that $v = 2.47 \times 10^{-18}$ cc. This is 5,000 times the volume of the molecule of oleic acid[9] or 60 times that of the molecule of egg albumin.[10] If combined trypsin is inactivated at all, the rate, compared to that of free trypsin, is very small. According to the theory given above, v must be correspondingly small. It need not be absurdly small, however; if it were equal to the volume of the molecule of oleic acid, nearly 3 years would be required for half-inactivation.

Two hypotheses may now be considered—that inactivation results (1) from the loss of an electron and (2) from the gain of an electron. The theory given above holds good in either case. That free trypsin is believed to exist as a positive monovalent ion[11] may help us to make a choice. There is no reason for supposing that the actual volume of the trypsin radical is different in the free and combined states. The probability that a beta particle will knock an electron out of this radical should then be independent of state, and consequently, on the first hypothesis, free and combined trypsin should be inactivated at the same rate. On the second hypothesis (inactivation by gaining an electron), the probability may not be independent of state. By virtue of its positive charge, the trypsin radical in the free state is doubtless able to capture a free electron from a considerable distance, whereas positive ions appearing in the neighborhood would be repelled. v would then be the volume of the sphere of influence of the ion. The negligible inactivation of combined trypsin may mean that in this condition the radical is neutralized electrically; that it makes, therefore, no attempt to appropriate free electrons which appear in or near it.

This second hypothesis, together with the assumption that trypsin in combination is electrically neutral, explains the facts qualitatively. Inactivation of free trypsin appears, therefore, to result from electrical neutralization of the ion by the addition of one electron.

[9] Langmuir, I., *J. Am. Chem. Soc.*, 1917, xxxix, 1848.

[10] du Noüy, P. L., *J. Biol. Chem.*, 1925, lxiv, 595.

[11] Northrop, J. H., *J. Gen. Physiol.*, 1923–24, vi, 337.

SUMMARY.

1. The inactivating effect of soft x-rays on trypsin in solutions of various degrees of concentration has been studied.

2. It has been found to run parallel with spontaneous heat inactivation. It is almost, if not entirely, confined to the free or active trypsin.

3. Under radiation of constant intensity, the inactivation follows the simple exponential law which indicates a monomolecular reaction.

4. Estimates have been made of the amount of ionization required to inactivate trypsin to half value in these experiments and in those of Hussey and Thompson, who employed the beta rays from Radium B and C. The close agreement corroborates the idea that the effect is a function of ionization only.

5. The nature of the process of inactivation is discussed; inactivation seems to result from electrical neutralization of the trypsin ion.

SOME CONSEQUENCES OF THE THEORY OF MEMBRANE EQUILIBRIA.

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The theory of membrane equilibria, as developed by Donnan,[1] has been shown by Loeb[2] to be the basis for a satisfactory explanation of the colloidal behavior of proteins. It seemed of interest to formulate, in more detail than Donnan has done, some of the consequences of this theory as applied to systems simpler than those studied by Loeb; namely, systems containing a non-diffusible base, acid, or ampholyte of known ionization constants.

The theory applies wherever one type of ion in a mixture of electrolytes is prevented from diffusing freely to all parts of the system, by some constraint such as a membrane impermeable to it but permeable to other ions. Donnan showed that in such systems there will, in general, be an unequal distribution of the diffusible ions between the solutions on the two sides of the membrane. The ratio of the activities of any diffusible ion in the two solutions must be equal to that of any other diffusible ion of the same sign and valence, and equal to the inverse ratio of the activities of any ion of the opposite sign but of the same valence. Where ratios of ions of different valence are compared, the same law holds if each ratio is raised to a power equal to the reciprocal of the valence of the ion.

As a consequence of this unequal distribution of ions, Donnan showed that a difference in electrical potential must exist between two such solutions, and that its magnitude must be given by the Nernst formula, being proportional to the logarithm of the ion ratio. He pointed out that a further consequence of this unequal distribution

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[1] Donnan, F. G., *a, Z. Elektrochem.*, 1911, xvii, 572; *b, Chem. Rev.*, 1924, i, 73.

[2] Loeb, J., Proteins and the theory of colloidal behavior, New York and London, 1st edition, 1922; 2nd edition, 1924.

must be a difference in osmotic pressure between the two solutions. Such differences in electrical potential and in osmotic pressure were measured by Loeb in experiments with various proteins, and were shown to be quantitatively in accord with the theory.

A derivation of Donnan's equations which is, perhaps, more rigorous, has been given by Hückel.[3] He pointed out that the original equations in terms of ion concentrations are exact for ideal dilute solutions, but that for real solutions the total concentration of each ion must be multiplied by its activity coefficient to give the ionic activity. This was previously recognized by Donnan.[1,b] Hückel also pointed out that, for non-ideal solutions, each concentration term in the osmotic pressure equation must be multiplied by an osmotic coefficient which is a function of the activity coefficient of the water in the solution.

In the equations which follow, ion concentrations are used in place of activities, with the understanding that the equations can be exact only for solutions so dilute that these quantities become identical. The equations are also based on the assumption of complete ionization for strong electrolytes. The cases to be considered are the addition of a strong acid or base to a system containing a weak non-diffusible base, acid, or ampholyte. The equations are based on (1) Donnan's equation for the ion ratio, (2) the constancy of the ion product for water, (3) the validity of the law of mass action for the ionization of the non-diffusible electrolyte, (4) the principle of electrical neutrality for each solution.

The equilibrium state for such systems may be represented by the following diagram, in which the capital letters represent molecular or ionic species, and the small letters their molar concentrations. The membrane is represented by the vertical line. The non-diffusible species are R, R^+, R^-. The valence of the cation of the base added is q, that of the anion of the acid is p.

[3] Hückel, E., Zsigmondy Festschrift, Dresden, 1925, 204. (Ergänzungsband, *Kolloid-Z.*, xxxvi, 204.)

$$
\begin{array}{r|l}
c - w - z \; \text{R} & \\
z \; \text{R}^+ & \\
w \; \text{R}^- & \\
y \; \text{H}^+ & \text{H}^+ \; x \\
v \; \text{OH}^- & \text{OH}^- \; u \\
\dfrac{n}{q} \; \text{B}^{q+} & \text{B}^{q+} \; \dfrac{m}{q} \\
\dfrac{1}{p}\,(y + z + n - w - v) \; \text{A}^{p-} & \text{A}^{p-} \; \dfrac{1}{p}\,(x + m - u)
\end{array}
$$

The condition of electrical neutrality is implied in the values for the concentration of the ion A^{p-}. The other conditions are the Donnan equation, the ion product equation, and the ionization equations, which are as follows:

$$
\lambda = \frac{x}{y} = \frac{v}{u} = \sqrt[q]{\frac{m}{n}} = \sqrt[p]{\frac{y + z + n - w - v}{x + m - u}}; \tag{1}
$$

$$
k_w = xu = yv; \tag{2}
$$

$$
k_a = \frac{yw}{c - w - z}; \tag{3}
$$

$$
k_b = \frac{vz}{c - w - z}. \tag{4}
$$

λ is Donnan's symbol for the ion ratio; k_w, k_a, and k_b have their usual significance. To these should be added the osmotic pressure equation

$$
P = RTe \tag{5}
$$

where e represents the difference in total concentration of molecules and ions in the two solutions. The equations which follow are all derived from these five, as applied to the special cases indicated.

I. Addition of a Monobasic Acid to a Non-Diffusible Base.

Here the values of w, k_a, m, and n are zero, while $p = 1$. Equation (1) becomes

$$
\lambda = \frac{x}{y} = \frac{v}{u} = \frac{y + z - v}{x - u} = \frac{y + z}{x}. \tag{6}
$$

(The last member results from the application of the laws of proportion to the two preceding it.) Since $x = \lambda y$, this may be written

$$\lambda = \sqrt{1 + \frac{z}{y}}. \tag{7}$$

For this case, by substituting $\frac{k_w}{y}$ for v and K for $\frac{k_w}{k_b}$, equation (4) becomes

$$\frac{z}{c - s} = \frac{y}{K} \quad \text{or} \quad z = \frac{cy}{K + y}. \tag{8}$$

From equations (7) and (8),

$$\lambda = \sqrt{1 + \frac{c}{K + y}}. \tag{9}$$

From this it appears that as the concentration of hydrogen ion is increased the value of the ion ratio must always decrease, approaching unity as a limit. Accordingly the initial rise in the membrane potential which Loeb observed on adding acid to isoelectric protein would not occur in the case of a non-amphoteric base.

This is not in agreement with the conclusion reached by Donnan[1,b] for this case, because he assumed that in the absence of acid the ionization of the base could be neglected. His reasoning would be strictly correct for the case of a non-diffusible substance forming no ions whatever in pure water, but forming ions by combination with H+ ion from the added acid. This condition is approximately fulfilled by the amphoteric proteins studied by Loeb, which furnish no excess of positive or negative ions at their isoelectric points, and hence do show the initial rise of the membrane potential on the addition of acid. If, however, a non-amphoteric base is appreciably ionized in pure water, its membrane potential should have a high value and could only be decreased by the addition of acid.

This can be made clear by proving that the derivatives $\frac{d\lambda}{dx}$ and $\frac{d\lambda}{dy}$ must always be negative. Inspection of equation (9) shows that $\frac{d\lambda}{dy}$ is always negative. Since $\lambda = \frac{x}{y}$, $\frac{d\lambda}{dy} = \frac{1}{y}\frac{dx}{dy} - \frac{x}{y^2}$. Since $\frac{d\lambda}{dy}$ is negative, $\frac{x}{y} > \frac{dx}{dy}$. But $\frac{d\lambda}{dx} = \frac{1}{y} - \frac{x}{y^2}\frac{dy}{dx}$. If this derivative is posi-

tive or zero, then $\frac{1}{y} \geqq \frac{x}{y^2}\frac{dy}{dx}$, and $\frac{dx}{dy} \geqq \frac{x}{y}$. But this has just been shown

to be false, and therefore $\frac{d\lambda}{dx}$ must be negative. Moreover $\frac{dy}{dx}$ and

$\frac{dx}{dy}$ are always positive, since they are quotients of the two negative

derivatives $\frac{d\lambda}{dx}$ and $\frac{d\lambda}{dy}$.

For the special case when the acid and base are present in equivalent amounts, equation (9) assumes a simpler form. If the volumes of the two solutions are kept equal, this condition of equivalence gives the equation

$$x + y + z = c + u + v.$$

Since the solution containing such a salt is acid, the value of v is negligible as compared with y, and since $\lambda > 1$, u is even smaller than v and may likewise be neglected. Thus the equation becomes, approximately,

$$c = x + y + z. \tag{10}$$

From equations (8) and (10),

$$c = \frac{(K + y)z}{y} = x + y + z, \text{ and } z = \frac{y(x + y)}{K}.$$

From equation (6), $z = \frac{x^2}{y} - y = \frac{(x - y)\,(x + y)}{y}$. Equating these

values of z, $\frac{y}{K} = \frac{x - y}{y} = \lambda - 1$, and

$$\lambda = 1 + \frac{y}{K}. \tag{11}$$

The osmotic pressure difference in the general case of a system containing a non-diffusible base and a strong acid should be given, as Donnan[1, b] has pointed out, by equation (5) in the form

$$P = RTe = RT(c + 2y + z - 2x). \tag{12}$$

Thus $e - c$ represents the difference between the concentrations of diffusible ions in the two solutions, and it is on this difference that the

changes in osmotic pressure with pH are dependent. The nature of these changes with pH may be inferred from the sign of the derivative of $e - c$.

From equation (6), $y + z = \dfrac{x^2}{y}$, and $\lambda = \dfrac{x}{y}$; hence

$$e - c = y + z - 2x + y = y(\lambda - 1)^2, \tag{13}$$

$$\frac{d(e - c)}{dy} = \frac{de}{dy} = (\lambda - 1)\left(\lambda - 1 + 2y\frac{d\lambda}{dy}\right). \tag{14}$$

From equation (9) it is evident that $\lambda > 1$ (except that when y becomes infinite, $\lambda = 1$); hence $\lambda - 1$ is positive. But since $\dfrac{d\lambda}{dy}$ is negative, the value of $\dfrac{de}{dy}$ will be positive if $\lambda - 1 > -2y\dfrac{d\lambda}{dy}$, negative if $\lambda - 1 < -2y\dfrac{d\lambda}{dy}$, and zero if $\lambda - 1 = -2y\dfrac{d\lambda}{dy}$. From equation (9), by differentiation,

$$\frac{d\lambda}{dy} = -\frac{c}{2\lambda(K + y)^2}.$$

Therefore $\dfrac{de}{dy}$ is positive when $\lambda - 1 > \dfrac{cy}{\lambda(K + y)^2}$. But since, by equation (9), $\dfrac{cy}{(K + y)^2} = \dfrac{(\lambda^2 - 1)y}{K + y}$, this condition becomes $(\lambda - 1)\lambda > \dfrac{(\lambda^2 - 1)y}{K + y}$, $\lambda > \dfrac{(\lambda + 1)y}{K + y}$, $\dfrac{K + y}{y} > \dfrac{\lambda + 1}{\lambda}$, $\dfrac{K}{y} > \dfrac{1}{\lambda}$ or $\lambda > \dfrac{y}{K}$. Also $\dfrac{de}{dy}$ is negative when $\lambda < \dfrac{y}{K}$ and zero when $\lambda = \dfrac{y}{K}$. In the latter case $y = \sqrt{Kx}$. This is the condition for a maximum and not a minimum of osmotic pressure, for when y is small, λ has its largest value, as has already been pointed out. Hence an increase in y will cause $\dfrac{de}{dy}$ and hence $\dfrac{dP}{dy}$ to have decreasing positive values, to pass through zero, and finally to have increasing negative values. Thus a rise and fall of the osmotic pressure with increasing acid, such as Loeb observed

with proteins, should also be found in the case of a non-amphoteric base.

II. Addition of a Monoacid Base to a Non-Diffusible Acid.

In this case the equations assume a form similar to those already given if negative ions are substituted for positive, $\dfrac{1}{\lambda}$ for λ, and k_a for k_b. They may be written as follows:

$$\frac{1}{\lambda} = \frac{u}{v} = \frac{y}{x} = \frac{v + w - y}{u - x} = \frac{v + w}{u}; \tag{6a}$$

$$\frac{1}{\lambda} = \sqrt{1 + \frac{w}{v}}; \tag{7a}$$

$$\frac{w}{c - w} = \frac{v}{K'} \quad \text{or} \quad w = \frac{cv}{K' + v}; \tag{8a}$$

$$\frac{1}{\lambda} = \sqrt{1 + \frac{c}{K' + v}}. \tag{9a}$$

Here K' is written in place of $\dfrac{k_w}{k_a}$. Equation (9a) shows that as the alkalinity is increased the value of $\dfrac{1}{\lambda}$ must decrease, or λ must increase, approaching 1 as a limit. The remaining equations for this case will be omitted, as they are exactly similar to those for the non-diffusible base if the above substitutions are made.

III. Addition of a Monobasic Acid to a Non-Diffusible Ampholyte.

In this case the values of m and n are zero, while $p = 1$. Equation (1) becomes

$$\lambda = \frac{x}{y} = \frac{v}{u} = \frac{y + z - w - v}{x - u} = \frac{y + z - w}{x}. \tag{15}$$

Substituting λy for x,

$$\lambda = \sqrt{1 + \frac{z - w}{y}}. \tag{16}$$

By solving equations (3) and (4) for z and w, substituting $\dfrac{k_w}{y}$ for v and K for $\dfrac{k_w}{k_b}$, it follows that

$$z = \frac{k_b c y}{k_b y + k_w + k_a v} = \frac{c y^2}{y^2 + K y + K k_a} \tag{17}$$

and

$$w = \frac{k_a c v}{k_a v + k_w + k_b y} = \frac{K k_a c}{y^2 + K y + K k_a}. \tag{18}$$

Hence

$$z - w = \frac{c(y^2 - I^2)}{y^2 + K y + I^2} \tag{19}$$

if $I^2 = K k_a = \dfrac{k_a k_w}{k_b}$, which means that I is the value of y at the isoelectric point of the ampholyte. From equations 16 and 19,

$$\lambda = \sqrt{1 + \frac{c(y^2 - I^2)}{y(y^2 + K y + I^2)}}. \tag{20}$$

(This becomes identical with equation (9) if $k_a = 0$.) From this it appears that at the isoelectric point, where $y = I$, $\lambda = 1$. On the acid side, where $y > I$, $\lambda > 1$, but in excess acid, where I^2 becomes negligible in comparison to y^2, then

$$\lambda = \sqrt{1 + \frac{c}{K + y}},$$

which is equation (9). Hence in excess acid the value of λ decreases with increase in y and again approaches 1 as a limit. (It is to be remembered that λ cannot be negative, since it is the ratio of two positive quantities, x and y.) Thus as increasing amounts of acid are added to the pure ampholyte solution, the value of λ must rise from 1 to a maximum and again decrease toward 1. This predicts a rise and fall of the membrane potentials, such as were observed by Loeb when increasing amounts of acid were added to isoelectric protein solutions.

The value of y corresponding to the maximum value of λ can be calculated by differentiating equation (20) and placing the derivative equal to zero. The resulting equation of condition is

$$y^4 - I^4 = 4I^2y^2 + 2I^2Ky, \tag{21}$$

and this can be solved graphically[4] for y if the values of K and I (or of k_a, k_b, and k_w) are known.

A rough test of this equation was made in the case of gelatin. It was shown in a previous paper[5] that the combination curve of gelatin with hydrochloric acid was approximately equivalent to the ionization curve of a monoacid base having $K = \dfrac{k_w}{k_b} = 2.4 \times 10^{-4}$. Using this value, together with $I = 2 \times 10^{-5}$ (pH = 4.7), a solution of equation (20) gave the value $y = 6.7 \times 10^{-5}$, or pH = 4.17. Loeb's observed maximum value of the membrane potential occurred at pH 4.0.

The osmotic pressure may be calculated, as before, by taking the algebraic sum of the concentrations.

$$P = RTe = RT(c + 2y + z - w - 2x). \tag{22}$$

By combining this with equation (16), it follows that

$$e - c = y\,(\lambda - 1)^2,$$

which is the same as equation (13).

With regard to the location of the maximum of osmotic pressure, the reasoning already given for the case of a non-amphoteric base will apply if the maximum occurs at such a high H^+ ion concentration that $w = 0$, a condition which makes equation (16) equivalent to equations (7) and (9). If this is the case, then $y = \sqrt{Kx}$, $\log K = 2 \log y - \log x$, or $pK = 2\,py - px$ (where p is Sörensen's symbol for $-$ log).

Loeb's data for gelatin chloride show that the maximum osmotic pressure was observed where $py = 3.33$ and $px = 2.87$. Hence $pK = 6.66 - 2.87 = 3.79$, while the value obtained from the writer's combination curve for gelatin and hydrochloric acid was $pK = 3.625$.

[4] Mellor, J. W., Higher mathematics for students of chemistry and physics, London and New York, 4th edition, 1919, 355.

[5] Hitchcock, D. I., J. Gen. Physiol., 1921–22, iv, 733.

The maximum value of Loeb's "Donnan correction" or calculated osmotic pressure, which depends on y $(\lambda - 1)^2$, occurred where $py = 3.25$ and $px = 2.81$; hence the value for pK should be $6.50 - 2.81 = 3.69$. This indicates that the simplified theory applies fairly well even in the case of a complex ampholyte such as gelatin.

The complete equation of condition for the maximum of osmotic pressure for an ampholyte with added acid is so complex as to be practically useless. It may be written

$$\lambda(\lambda - 1) = \frac{c}{y} \frac{y^4 - 4I^2y^2 - 2I^2Ky - I^4}{(y^2 + Ky + I^2)^2}.$$

IV. Addition of a Monoacid Base to a Non-Diffusible Ampholyte.

In this case $q = 1$, while the concentration of the ion A^{p-} is zero. Hence $w + v = y + z + n$ and $u = x + m$. Equation (1) becomes

$$\frac{1}{\lambda} = \frac{y}{x} = \frac{u}{v} = \frac{n}{m} = \frac{w + v - y - z}{u - x} = \frac{v + w - z}{u}. \tag{23}$$

Substituting $\dfrac{v}{\lambda}$ for u,

$$\frac{1}{\lambda} = \sqrt{1 + \frac{w - z}{v}} = \sqrt{1 + \frac{(w - z)\,y}{k_w}}. \tag{24}$$

Combining this with equation (19),

$$\frac{1}{\lambda} = \sqrt{1 + \frac{cy\,(I^2 - y^2)}{k_w\,(y^2 + Ky + I^2)}}. \tag{25}$$

(This becomes identical with equation (9a) if $k_b = 0$.) At the isoelectric point, $y = I$, and $\dfrac{1}{\lambda} = 1 = \lambda$. With increasing alkali, y becomes smaller than I, so that the fraction in equation (25) has finite positive values and $\dfrac{1}{\lambda} > 1$ or $\lambda < 1$. With more alkali y^2 becomes negligible, and equation (25) takes the form

$$\frac{1}{\lambda} = \sqrt{1 + \frac{k_a cy}{k_w(k_a + y)}},$$

which is identical with equation (9a). Finally, as y is decreased still more, $\frac{1}{\lambda}$ decreases, approaching 1 as a limit. Thus with increasing alkali the values of λ, starting at 1, decrease, pass through a minimum, and finally increase, approaching 1.

The position of the minimum can be calculated as before by differentiation. Again the calculation involves the graphical solution of the equation of condition, which is

$$y^4 + 2Ky^3 + 4I^2y^2 = I^4. \tag{26}$$

This equation probably could not be applied, even roughly, to experiments with proteins, since their titration curves show that they have, in general, two sets of acid groups, or two mean acidic ionization constants.

In this case the osmotic pressure is given by

$$P = RTe = RT(c + 2v + w - z - 2u). \tag{27}$$

From equation (24), $v + w - z = \dfrac{v}{\lambda^2}$.

Hence

$$e - c = v\left(\frac{1}{\lambda^2} - \frac{2}{\lambda} + 1\right) = v\left(\frac{1}{\lambda} - 1\right)^2. \tag{28}$$

Again the complete equation of condition for the maximum of osmotic pressure is very complicated, but if the maximum occurs at a sufficiently high alkalinity so that z becomes negligible, the calculation may be made as for a non-amphoteric acid. Thus equation (24) becomes identical with equations (7a) and (9a). By reasoning similar to that in Section I it can be shown that for this case the condition for the maximum of osmotic pressure is that $\dfrac{1}{\lambda} = \dfrac{v}{K'}$, $\lambda = \dfrac{y}{k_a}$ or $y = \sqrt{k_a x}$.

V. Addition of Acid and Salt to a Non-Diffusible Base.

In systems containing ions of different valence, the equations differ in form according to the valence of the ions. Considering first the case of a non-diffusible base ROH with the addition of a monobasic acid, as HCl, and salts having the same anion but cations of variable

valence q, the conditions imposed upon equation (1) are that $p = 1$ and $w = 0$. Accordingly

$$\lambda = \frac{x}{y} = \frac{v}{u} = \sqrt[q]{\frac{m}{n}} = \frac{y + z + n - v}{x + m - u} = \frac{y + z + n}{x + m}. \tag{29}$$

For the special cases where there is no salt added, and where the salt has a cation of valence 1, 2, or 3, the following equations give the value of the ion ratio:

ROH, HCl:　　　$\lambda = \sqrt{1 + \dfrac{z}{y}} = \sqrt{\dfrac{y + z}{y}};$　　　(7)

ROH, HCl, NaCl:　$\lambda = \sqrt{1 + \dfrac{z}{y + n}} = \sqrt{\dfrac{y + z + n}{y + n}};$　　　(30)

ROH, HCl, CaCl$_2$:　$\lambda = \sqrt{\dfrac{y + z + n}{y + \lambda n}} = \sqrt{\dfrac{y + z + n}{y + \sqrt{mn}}};$　　(31)

ROH, HCl, LaCl$_3$:　$\lambda = \sqrt{\dfrac{y + z + n}{y + \lambda^2 n}} = \sqrt{\dfrac{y + z + n}{y + \sqrt[3]{m^2 n}}}.$　　(32)

If the values of λ for these systems are compared for identical values of y and n, it will be observed that the addition of any salt decreases the value of λ. This is in accord with the observations of Loeb in the case of gelatin and other proteins. Loeb also found, in comparing the effect of the three salts mentioned at the same equivalent concentrations and the same pH, that the values of λ were identical. Equations (30), (31), and (32) can be identical only when $\lambda = 1$ or $m = n$. It is possible that some differences due to the valence of the cation might be detected with more refined experimental methods.

If sulfuric acid and sulfates are used in place of hydrochloric acid and chlorides, the value of p is 2, and equation (1) becomes

$$\lambda = \frac{x}{y} = \frac{v}{u} = \sqrt{\frac{m}{n}} = \sqrt{\frac{y + z + n - v}{x + m - u}}. \tag{33}$$

If the hydroxyl ion concentrations are neglected, the following equations should give the ion ratio for the special cases indicated:

ROH, H$_2$SO$_4$:　　　$\lambda = \sqrt[3]{1 + \dfrac{z}{y}} = \sqrt[3]{\dfrac{y + z}{y}};$　　　(34)

$\text{ROH, H}_2\text{SO}_4\text{, Na}_2\text{SO}_4\text{:} \quad \lambda = \sqrt[3]{1 + \dfrac{z}{y + n}} = \sqrt[3]{\dfrac{y + z + n}{y + n}};$ (35)

$\text{ROH, H}_2\text{SO}_4\text{, MgSO}_4\text{:} \quad \lambda = \sqrt[3]{\dfrac{y + z + n}{y + \lambda n}} = \sqrt[3]{\dfrac{y + z + n}{y + \sqrt{mn}}};$ (36)

$\text{ROH, H}_2\text{SO}_4\text{, La}_2(\text{SO}_4)_3\text{:} \quad \lambda = \sqrt{\dfrac{y + z + n}{y + \lambda^2 n}} = \sqrt[3]{\dfrac{y + z + n}{y + \sqrt[3]{m^2 n}}}.$ (37)

These equations are similar to the preceding group in the expressions under the radical signs, and accordingly here again there should theoretically be some differences in λ due to the valence of the cation. Such differences, however, must be slight in comparison with the difference caused by taking the cube root instead of the square root, which is due to the valence of the anion. This difference, as Loeb has pointed out, makes the value of the membrane potential in the case of the bivalent anion just two-thirds of that observed in the case of the univalent anion.

The eight equations just mentioned assume the following general form, if p represents the valence of the common anion and q the valence of the cation of the salt:

$$\lambda = \sqrt[p+1]{\dfrac{y + z + n}{y + \lambda^{q-1} n}} = \sqrt[p+1]{\dfrac{y + z + n}{y + \sqrt[q]{m^{q-1} n}}}.$$ (38)

SUMMARY.

In applying Donnan's theory of membrane equilibria to systems where the non-diffusible ion is furnished by a weak acid, base, or ampholyte, certain new relations have been derived. Equations have been deduced which give the ion ratio and the apparent osmotic pressure as functions of the concentration and ionization constant of the weak electrolyte, and of the hydrogen ion concentration in its solution. The conditions for maximum values of these two properties have been formulated. It is pointed out that the progressive addition of acid to a system containing a non-diffusible weak base should not cause the value of the membrane potential to rise, pass through a maximum, and fall, but should only cause it to diminish. It is shown that the theory predicts slight differences in the effect of salts on the ion ratio in such systems, the effect increasing with the valence of the cation.

NOTES ON MICROSPECTRA.

I. The Absorption Spectrum of Euglena.

By LOURENS G. M. BAAS-BECKING and P. A. ROSS.

(From the Department of Physiology, Stanford University, Stanford.)

The opacity or transparency of a certain substance for monochromatic radiation plotted against wave-length yields a so called absorption or transmission curve. The finite integral of this function between two definite wave-lengths represents a certain fraction of the corresponding integral for the source of light used. This fraction is the percentage of energy absorbed (absorption curve) or transmitted (transmission curve) by the absorbing substance. The absorption curves are therefore sometimes incorrectly called energy curves.

The absorption curve of the living leaf has been determined, more or less accurately, by various authors. The best and most recent values available are those of Ursprung,[1] in whose paper the older literature will be found. Ursprung used a linear thermocouple to measure the absorption of green leaves (*Acer, Tradescantia*) at various places of the spectrum (10 $\mu\mu$ apart).

The ideal object for the study of these absorption curves would be the isolated plastid, excluding the optical interference of air, water, cellulose walls, and other substances. Certain euglenids approximate this condition.

With the aid of an Abbe prismatic microspectral ocular (E. Leitz), which had been provided with adjustable slits for both absorption and comparison spectra, the authors were able to obtain spectrum photographs of minute objects (limit 10 $\times$ 15 μ). The source of light used was a 6 volt "Mignon" lamp which ran on a battery. An Eastman panchromatic film was used throughout. The procedure was simple. Exposures were taken with and without the organisms in the micro-

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[1] Ursprung, A., *Ber. bot. Ges.*, 1918, xxxvi, 73.

scopic field, the photographs developed with pyro developer in ab-
solute darkness.

The opacity of the negatives for various regions of the spectrum was
determined with the aid of a modified Boys' radiomicrometer.[2]   This
combination thermocouple and galvanometer was constructed in the
following way: Soldered on to a strip of silver foil (12 mm².) are a thin
silver wire, and at the other corner, a thin bismuth wire.   The free
ends of the wires are joined to a thin copper coil, which serves as
galvanometer coil and to which a galvanometer mirror is attached.
The resistance of the copper coil about equals the resistance of the
rest of the system.   The apparatus is steadied by a glass capillary and
suspended on a quartz fiber.   The coil is brought between the poles of
a powerful permanent magnet.   The silver foil is blackened by three
coats of India ink, the entire apparatus properly insolated.

A constant light source was obtained by running a 6 volt lamp on
a 104 volt storage battery current with tungsten lamps (which illu-
minated the scale) in series.   The film moved slowly over an adjustable
slit.

The density of the plate is proportional to the logarithm of the re-
ciprocal galvanometer deflection.   Adjustment of the nul point by
correspondence of the fog reading on the film to 100 scale divisions
simplifies the calculations materially.   The spectra were measured at
intervals of .7 mm.; at critical points intermediate readings (down
to .07 mm.) were taken.   The Amici prism was calibrated with the
aid of three Fraunhofer lines of the solar spectrum with Hartmann's
simplified interpolation formula.[3]   This formula expresses the wave-
length λ as a function of a linear distance d as follows:

$$\lambda = \lambda_0 + \frac{c}{d_0 - d}$$

in which c and d_0 are constants; d represents the distance of the lines
measured.   The position of the Fraunhofer lines 486 $\mu\mu$ (H), 527 $\mu\mu$
(Fe) and 589 $\mu\mu$ (Na) was established on the radiomicrometer curve

[2] Boys, C. V., *Phil. Tr.*, *Series A*, 1889, clxxx, 159, 183.   See also Paschen, F.,
Ann. Phys., 1893, xlviii, 272.

[3] Hartmann, J., *Astrophys. J.*, 1898, viii, 218.

of a solar spectrum. Taking 486 $\mu\mu$ as an arbitrary zero we found

λ	d
486$\mu\mu$	= 0
527	= 18.8
589	= 39.4 divisions.

The constants were determined with the aid of these three sets of data

$$486 = \lambda_0 + \frac{c}{d_0} \tag{1}$$

$$527 = \lambda_0 + \frac{c}{d_0 - 18.8} \tag{2}$$

$$589 = \lambda_0 + \frac{c}{d_0 - 39.4} \tag{3}$$

The constants proved to be:

$$\lambda_0 = 214.9$$
$$d_0 = 143.1$$
$$c = 38794.4$$

The distance between 486 $\mu\mu$ and the maximum film density in the red for a *Euglena* spectrum proved to be 57.8 divisions.   The wave-length of the absorption maximum was therefore determined at

$$\lambda = 214.9 + \frac{38794.4}{143.1 - 57.8} = 214.9 + 454.8 = 669.7 \ \mu\mu$$

The low dispersion in the red increases the probable error.   The value is very close to that obtained by other authors.

Ursprung's curve was transformed to the same abscissæ, the ordinates expressing transparency instead of extinction. Ursprung's curve comprises 24 determinations, in most cases 10 $\mu\mu$ apart, in the range investigated by us. (See Fig. 1.)

The nature of our negative allowed for 56 determinations in the same region (700–450 $\mu\mu$) for *Euglena*.   With good negatives the number of determinations could easily be increased tenfold.   The following table gives the location of the absorption maxima for *Euglena* as compared with Willstätter's (visual) determination for a living leaf.[4]

[4] Willstätter, R., and Stoll, A., Untersuchungen ueber Chlorophyll, Berlin, 1913, 62.

Data for *Euglena*.	Willstätter.
670$\mu\mu$	693–663$\mu\mu$
—	643
626 ⎫	
613 ⎭	625–611
586	592–569
543	551–535
528	520– end absorption.
476	
468	

No trace of band 643 $\mu\mu$ could be found. This is probably due to the steepness of the curve in this region. More determinations with
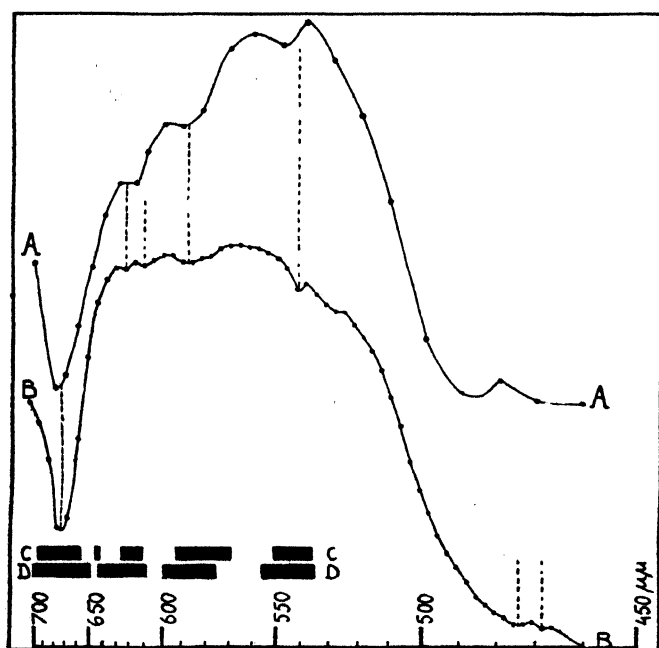
FIG. 1.

better negatives should reveal this band. The obvious advantage of our method is shown in the above table. The accompanying figure represents Ursprung's data (*A*), our data for *Euglena* (*B*), Willstätter's determination for the living leaf (*C*) and for an aqueous suspension of Chlorophyll *a* (*D*). The absorption bands are all drawn in black. The great similarity of Ursprung's and our curve is apparent.

TEMPERATURE AND THE MECHANISM OF LOCOMOTION IN PARAMECIUM.

By OTTO GLASER.

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I.

The effect of temperature on the velocity of forward movement in *Paramecium* is described accurately by the formula of Arrhenius, the values for μ being 8,000 in the upper ranges and 16,000 in the lower (Glaser, 1924–25). This result rests on the ordinary averages derived from the behavior of numerous individuals all belonging to a single pure line. At each temperature, however, averages mask the limits of variability; and these limits are highly significant in the precise study of thermal increments (Crozier and Federighi, 1924–25; 1925). For this reason a further analysis of the data on *Paramecium* is desirable. Moreover, for the temperatures considered by Crozier and Federighi, linear translation in *Oscillatoria* filaments, as well as the heart beat of *Bombyx* larvæ, yield thermal increments constant at 9,240 and 12,200 respectively. It is additionally interesting, therefore, to consider also a case in which averages indicate a sharp break in the neighborhood of 15°.

II.

The material available for this analysis consists of 709 records in which the time required for *Paramecium* to swim a constant distance at different temperatures was measured with a stop-watch.

Inasmuch as the details of these experiments have been fully described (Glaser, 1924–25) it is necessary to consider only the methods employed in the present handling of the data. The latter are influenced by three conditions each of which can be a source of artefacts. First, the number of observations is not constant over the entire range, and hence this number at each temperature must be

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reduced to a percentage basis. Second, the mass of readings now under consideration include all cases in which the temperature remained either absolutely constant or, in certain instances, changed by as much as 0.4°. This amount of latitude falls within the limits of experimental error, and was suggested when a preliminary plotting of data constant to within 0.1° disclosed for frequency curves not more than 1° apart, and including at least 30 points, no essential difference of spread. This property and the known constancy of the thermal increment (except at the "critical" point), together seemed to justify the formation of small temperature classes of which the largest has a mean spread of very much less than 0.4°.

Third, and more important than the treatment of the temperature records or of the number of observations, is that accorded the time measurements, because these exhibit a quality likely to have wider interest. With rising temperature the preliminary plots all indicated a marked decrease in the variability of the time element. This was evident not only from a progressive shrinkage in the width of the curves, but also from a corresponding increase in the density of points about the modes. There is, of course, no reason for doubting the correctness of this result. On the other hand there are certain considerations which make it understandable. As the temperature rises from 6° to 40° the time required to swim a unit distance decreases to about $\frac{1}{6}$ of its original value, whereas the reaction time of the experimenter remains constant. At the higher temperatures therefore an observer can easily record fewer differences than in the lower ranges where the time required for making the necessary discriminations is ample.

If the apparent decrease in the variability of the time factor inheres in the *Paramecium* and not in the conditions under which the records were made, it should persist in our curves when for time we substitute the logarithm of time. This procedure, accordingly, was adopted and is equivalent to the fiction that for all temperatures and all observations, the time available for making discriminations was the same.

The necessary statistical time classes on the logarithmic scale include all the times which differ by not more than 0.05 logarithmic units. The percentage frequencies of each class, plotted on the mid-ordinate of each time group give the frequency polygons shown in Fig. 1. The principle which guided these constructions is that they should
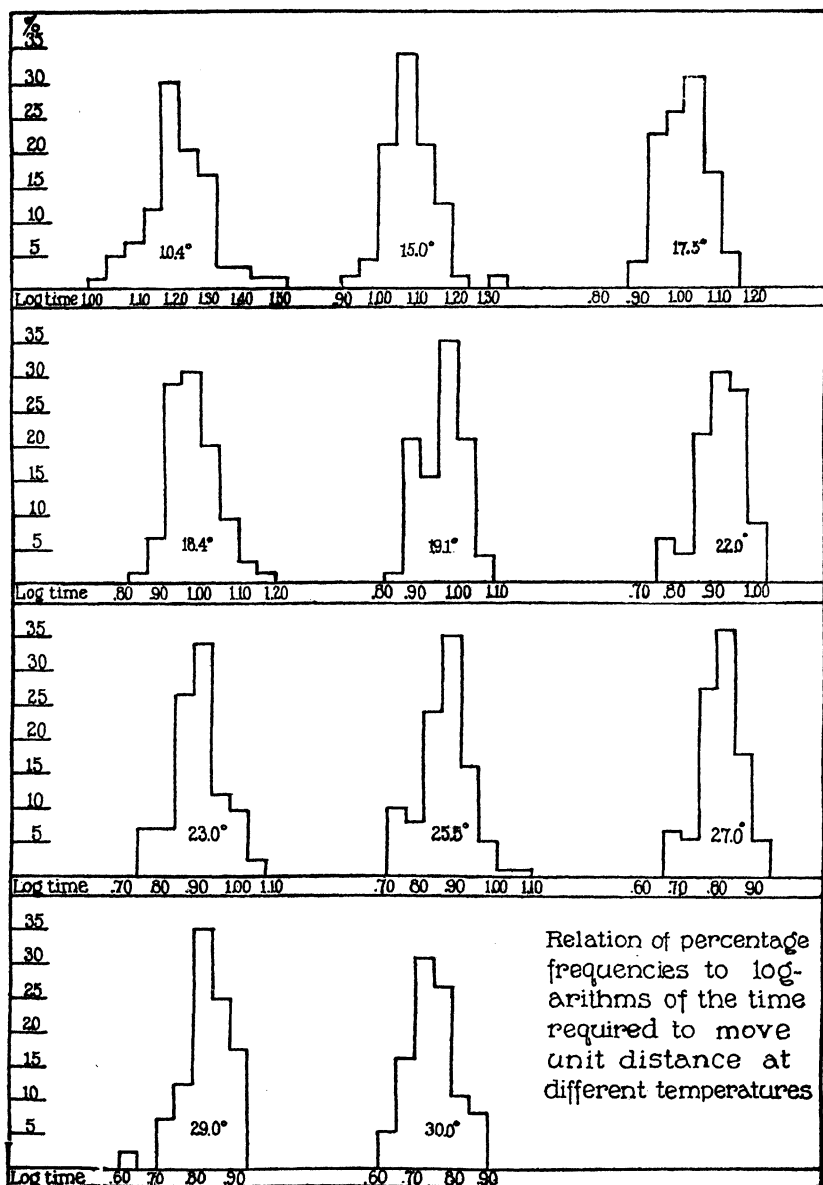
FIG. 1. Variation in time required to move unit distance at constant temperatures in *Paramecium*.

describe the points as closely as possible rather than that they should in all respects conform to statistical convention. As a matter of fact the most striking qualities of normal probability curves are clearly present; there is an evident approach to bilateral symmetry and inflection occurs on ordinates of standard deviation from the mean. However, the calculated "theoretical" maxima diverge somewhat from the maximal percentage frequencies actually found; yet until a "theoretical" maximum in biological statistics can be accredited in dynamic terms, it appears safer to assume that the greatest relative credibility attaches to the points representing the greatest number of observations.

TABLE I.

Tempera-ture.	No. of observa-tions.	Minimum.		Maximum.		Mode.		Mean.
		Frequency.	Log time.	Frequency.	Log time.	Frequency.	Log time.	Log time.
		per cent		per cent		per cent		
10.4	60	1.7	1.52	1.7	1.02	29.9	1.22	1.26
15.0	47	2.1	1.32	2.1	.92	34.1	1.07	1.08
17.5	60	3.4	1.14	3.4	.89	36.6	1.03	1.03
18.4	64	1.6	1.18	1.6	.83	30.4	.98	1.00
19.1	70	4.2	1.08	1.4	.83	35.0	.98	.94
22.0	46	8.7	1.01	6.5	.76	30.4	91	.91
23.0	41	2.4	1.06	7.2	.76	34.0	.91	.89
25.5	100	1.0	1.02 / 1.12	10.0	.72	35.0	.87	.85
27.0	72	5.5	.93	6.9	.68	35.9	.83	.81
29.0	40	17.5	.92	2.5	.62	35.0	.82	.83
30.0	109	8.2	.87	5.5	.62	30.9	.72	.74

It is desirable to check the validity of these curves in another way. If the groupings on which they rest do not distort the data, then log velocities deduced from the log time classes should behave in a predictable manner. Plotting their values according to Crozier's method (1924–25) against reciprocals of absolute temperature, should result, above 15°, in four straight lines, one for the means and one each for maxima, minima, and modes. These curves should be parallel and should have increments of 8,000. In an ideal case these values would fall between the lines describing maxima and minima respectively and these might be equidistant from the one describing the means. Below

15° the corresponding curves should give $\mu = 16,000$, though the distance between the maximal and minimal lines might easily exceed or be smaller than it is in the higher ranges. How closely these conditions are fulfilled is shown in Table I and Fig. 2.

It seems reasonable then to accept the frequency polygons as they stand. Without insisting that their value exceeds that of a useful approximation, we may conclude on the basis of spread and height that in the region above 15°, and again very possibly also below, the variability in the time required to swim a unit distance shows no tendency to rise or fall, but remains essentially constant.
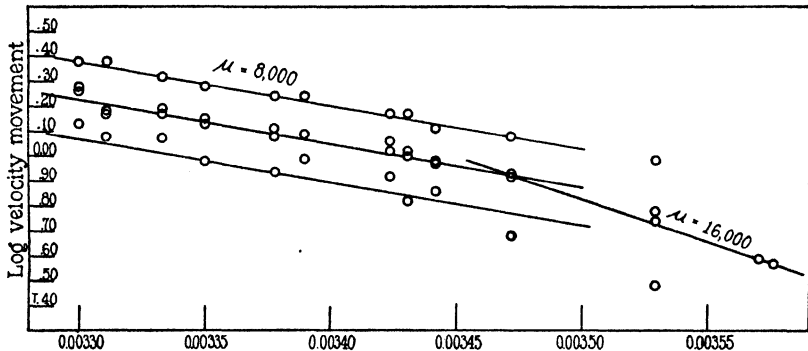
FIG. 2. Velocities deduced from log time classes, plotted against reciprocals of the absolute temperature. The line describing the modes is omitted. The two points at 7° and 6.6° are merely averages included to serve as guides. Below 15° there are not enough data at present to warrant the erection of maximal and minimal boundaries.

III.

Constancy above 15°, substantiated as it is, suggests an essential homogeneity of the material. Apparently a rise from the "critical" point to 30° does not seriously change the character of the system which controls the speed. This fact, for which evidence is now available, was merely an assumption necessary for the theoretical explanations offered in my previous paper. It is not surprising then that the facts of variability should easily harmonize with the earlier analysis based on averages alone.

The underlying mechanism assumed to account for this behavior of

Paramecium in relation to temperature is a chemical reaction system controlled by a catenary series in which 0, a source of original supply, is changed into an available form A, which on destruction to the end-product E, yields the energy that results in movement. It was suggested that for $0 \rightarrow A$ μ has a value of 8,000, and for $A \rightarrow E$, 16,000. Under these conditions a depletion of A is likely and would explain the decreased rate of acceleration at the higher temperatures. The case presented by the frequency polygons is in principle the same. We can suppose, with Loeb and Chamberlain (1915) and with Crozier and Federighi (1924–25), that differences in the velocity of different individuals and of the same individual at different times can be attributed to differences in the effective concentration of certain catalysts. This assumption merely transfers the control of $0 \rightarrow A$ and of $A \rightarrow E$ to catalytic agents either inevitably present during the passage of $0 \rightarrow A \rightarrow E$ or to catalysts in origin independent of the major reactants. If now the formation of such active catalysts has a temperature coefficient equal to that of the biological process under consideration—in the present instance, forward movement in *Paramecium*—it follows that the slowest individuals and the fastest, as groups, must have rates of acceleration equal to that of their mean, to which the extreme speeds should be related as constant fractions.

Above 15° this condition is closely approached by the polygons and by the curves in Fig. 2. Below 15° there is also some evidence indicating constancy in their relations, but whether the constants are the same must remain questionable until sufficient data are available for the lower range. Inasmuch as control at the lower temperatures by assumption rests with another catalyst, it might well be that its limits of variability in concentration are different from those of the catalytic agent controlling the reaction system above the "critical" point (*cf.* figures in Crozier and Stier, 1924–25).

The system of control then can be represented as

$$O \longrightarrow A \longrightarrow E$$
$$\mu_c = 8{,}000 \quad \mu_c = 16{,}000$$
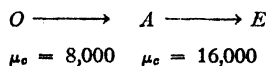
(μ_c pertaining to catalyst) which though slightly more complicated than $0 \rightarrow A \rightarrow E$ involves no change in the underlying conception. Moreover, analysis in terms of controlling catalysts in the long run

may result in great simplifications since the reactions and reacting substances in living things are innumerable, whereas the number of catalysts involved may possibly prove to be quite small.

SUMMARY.

1. Frequency polygons in which the number of observations at each temperature is reduced to a percentage basis while the time factor is represented on a logarithmic scale, indicate that the time required by *Paramecium* to swim a unit distance at different temperatures varies within definite limits which are constant above 15°. Below 15° the range of variability very possibly is not the same, though probably likewise constant.

2. Logarithmic velocities deduced from mean, maximal, minimal, and modal time classes, when plotted against reciprocals of the absolute temperature, fall respectively on straight lines. These lines are parallel and give μ values of 8,000 above 15° and probably 16,000 below.

3. This implies that the mechanism of locomotion in *Paramecium* remains essentially unaltered by a rise in temperature from 15° to 30°, and probably remains in a similar sense constant from 6° to 15°.

4. The theoretical interpretation of this result is possible in terms of a catenary series $0 \rightarrow A \rightarrow E$ in which the passages $0 \rightarrow A$ and $A \rightarrow E$ are controlled by two catalysts differing respectively in concentration in different individuals and perhaps at different times in the same individual, but depending for their effective concentration on reactions having temperature coefficients identical with those which at each temperature characterize the biological process under consideration.

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STUDIES ON SALT ACTION.

X. The Influence of Electrolytes upon the Viability and Electrophoretic Migration of Bacterium Coli.*

By HOWARD J. SHAUGHNESSY and KATHARINE I. CRISWELL.

(From the Department of Public Health, Yale School of Medicine, New Haven.)

INTRODUCTION.

Among the numerous contributions dealing with the effect of electrolytes upon bacterial physiology (exhaustively reviewed by Falk, 1923) there are two sets of studies which bear directly on the work here reported.

Holm and Sherman (1921) showed that the growth of *Bacterium coli* in 1 per cent peptone solution is accelerated by certain anions and by certain cations in .2 M concentration and retarded by others. Sodium chloride, KCl, and NH$_4$Cl were particularly favorable, Na fluoride and CaCl$_2$ particularly unfavorable. It was of special interest to note that NaCl and Na$_2$SO$_4$ widened the zone of hydrogen ion concentration within which the organism would develop, making its growth fairly rapid at pH 5.2 and at pH 8.2. On the other hand more toxic salts, such as Na citrate, showed an additive rather than an antagonistic effect, narrowing the zone of acidity and alkalinity within which the organisms would develop freely. Sherman and Holm (1922) and Sherman, Holm, and Albus (1922) confirmed and extended these results.

A second series of papers, from our own laboratory, has dealt with the same general problems. Hotchkiss (1923), using the method employed by Sherman and his colleagues, worked out the inhibitive concentration of twenty-three cations in union with chlorine. The

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* Based on an essay presented by the junior author in candidacy for the Certificate in Public Health at Yale University.

monovalent ions were in general least toxic, followed by the alkaline earth metals and then by the heavy metals. Fifteen of the twenty-three salts studied, including such toxic substances as $HgCl_2$, $CeCl_3$ and $ZnCl_2$, proved stimulating, rather than inhibitive, in very low concentration. Winslow and Falk (1923, a) obtained more detailed data on the influence of NaCl and $CaCl_2$ in varying concentration and at various pH values upon the viability of *Bacterium coli* in water. The quantitative effects in this case would naturally be expected to be quite different from those observed in a peptone culture medium. These observers found that .0145 M NaCl and .00145 M $CaCl_2$ were stimulating, while .725 M NaCl and .435 M $CaCl_2$ were distinctly toxic at all reactions. Calcium chloride in .145 M strength proved toxic only in an unadjusted alkaline solution, where its action was apparently due to interference with the normal power of the bacteria to adjust the reaction of a zone of contiguous liquid to a favorable pH value. In a second paper (Winslow and Falk, 1923, b) these investigators show that in a mixture of four parts of NaCl to one part of $CaCl_2$, with a total tonicity of .725 M, the inhibitive effect which either salt would by itself exert, disappears as a result of mutual antagonism. This antagonistic action, however, takes place only on the alkaline side of the neutral point.

In the present study we desired to repeat some of the work of Winslow and Falk and to supplement it by observation of the effect of what physiologists consider a more completely balanced solution, using for this purpose a Ringer-Locke solution (.155 M NaCl, .003 M KCl, and .002 M $CaCl_2$)—of slightly more than isotonic strength.

We also desired to compare the influences produced by the electrolytes studied upon viability with the effects exerted upon migration in the electrical field. Northrop and De Kruif (1921–22), Winslow, Falk, and Caulfield (1923–24) Winslow and Shaughnessy (1923–24), and Winslow and Fleeson (1925–26) have shown that electrolytes markedly influence electrophoretic charge; and Eggerth (1923–24) has suggested that such changes run more or less parallel to simultaneous changes in viability, at least in buffered solutions. The work of Northrop and De Kruif and of Winslow and Fleeson on the other hand strongly suggests that the effects produced on electrophoretic charge are only remotely connected with vital phenomena. There are, however, certain

suggestive parallelisms between the curves for cataphoresis and those for viability which made it seem worth while to test the point somewhat more fully.

Technique.

Our work was all carried out with a single strain of *Bacterium coli* isolated in this laboratory in 1924 from New Haven sewage. It was cultivated on standard nutrient agar in Kolle flasks for 20–24 hours at 37°C. The growth was washed off in distilled water, filtered through absorbent cotton, then centrifuged, and rewashed three times, always under sterile conditions. The final washed suspension was shaken with glass beads and seeded into flasks of the appropriate menstruum. The seeded flasks were kept for 4 hours at room temperature and plates were made immediately after the first seeding and at the end of the 1st, 2nd, and 4th hours. At each of these periods the reaction of each flask was readjusted to its original reaction, to compensate for the buffering influence of the bacterial cells, using the indicators of Clark and Lubs, at pH values 2.0, 6.0, and 8.0 and the indicators of Prideaux (1917) with Northrop and De Kruif's buffer solution at pH 11.0.

The tests of electrophoretic velocity were made according to the methods previously used in this laboratory and described by Winslow, Falk, and Caulfield (1923–24) and by Winslow and Fleeson (1925–26).

The fundamental menstrua used were distilled water, Ringer-Locke solution, sodium chloride in four concentrations (1.45 M, .725 M, .145 M, and .0145 M), and calcium chloride in the same four concentrations—each solution being studied at four different reactions (pH 2.0, pH 6.0, pH 8.0, and pH 11.0).

Results in Regard to Viability.

The results of our study, so far as viability is concerned, are presented in the form of averages in Tables I to X and in Figs. 1 and 2.

It is at once apparent from tables and charts that strongly acid solutions (pH 2.0) were highly toxic under all conditions, the vast majority of the bacteria being no longer viable at the end of the 1st hour. A highly alkaline solution (pH 11.0) was almost as deadly, except in distilled water where one-third of the bacteria survived for 2 hours (Table I).

TABLE I.

Viability of Bacterium coli in Distilled Water.

Hrs.	Per cent alive at pH			
	2	6	8	11
0	100.0	100.0	100.0	100.0
1	1.0	195.0	171.0	79.0
2	1.0	146.0	109.0	35.0
4	1.0	115.0	120.0	4.0
No. of experiments...	5	5	4	3

TABLE II.

Viability of Bacterium coli in Ringer's Solution.

Hrs.	Per cent alive at pH			
	2	6	8	11
0	100.0	100.0	100.0	100.0
1	1.0	101.0	73.0	0.0
2	2.0	107.0	141.0	0.0
4	1.0	198.0	65.0	0.0
No. of experiments...	4	5	5	3

TABLE III.

Viability of Bacterium coli in Sodium Chloride Solution. 1.45 M.

Hrs.	Per cent alive at pH			
	2	6	8	11
0	100.0	100.0	100.0	100.0
1	13.0	243.0	4.0	0.0
2	3.0	118.0	1.0	0.0
4	2.0	65.0	0.0	0.0
No. of experiments...	3	4	3	2

TABLE IV.

Viability of Bacterium coli in Sodium Chloride Solution. .725 M.

Hrs.	Per cent alive at pH			
	2	6	8	11
0	—	100.0	100.0	—
1	—	120.0	36.0	—
2	—	131.0	29.0	—
4	—	41.0	4.0	—
No. of experiments...	0	4	3	0

TABLE V.

Viability of Bacterium coli in Sodium Chloride Solution. .145 M.

Hrs.	Per cent alive at pH			
	2	6	8	11
0	—	100.0	100.0	—
1	—	90.0	88.0	—
2	—	49.0	58.0	—
4	—	88.0	112.0	—
No. of experiments...	0	3	3	0

TABLE VI.

Viability of Bacterium coli in Sodium Chloride Solution. .0145 M.

Hrs.	Per cent alive at pH			
	2	6	8	11
0	100.0	100.0	100.0	100.0
1	0.1	133.0	90.0	0.0
2	0.1	108.0	55.0	0.0
4	0.1	91.0	60.0	0.0
No. of experiments...	3	4	4	2

TABLE VII.

Viability of Bacterium coli in Calcium Chloride Solution.   1.45 M.

Hrs.	Per cent alive at pH			
	2	6	8	11
0	—	100.0	100.0	—
1	—	12.0	18.0	—
2	—	24.0	0.0	—
4	—	0.0	0.0	—
No. of experiments...	0	3	2	0

TABLE VIII.

Viability of Bacterium coli in Calcium Chloride Solution.   .725 M.

Hrs.	Per cent alive at pH			
	2	6	8	11
0	—	100.0	100.0	—
1	—	52.0	9.0	—
2	—	526.0	5.0	—
4	—	98.0	2.0	—
No. of experiments...	0	3	2	0

TABLE IX.

Viability of Bacterium coli in Calcium Chloride Solution.   .145 M.

Hrs.	Per cent alive at pH			
	2	6	8	11
0	—	100.0	100.0	—
1	—	155.0	50.0	—
2	—	151.0	8.0	—
4	—	64.0	10.0	—
No. of experiments...	0	3	2	0

TABLE X.

Viability of Bacterium coli in Calcium Chloride Solution. .0145 M.

Hrs.	Per cent alive at pH			
	2	6	8	11
0	100.0	100.0	100.0	100.0
1	0.3	130.0	173.0	0.0
2	1.0	40.0	50.0	0.0
4	0.4	65.0	45.0	0.0
No. of experiments...	2	3	3	2

In comparing the effect of the various salts at the two intermediate pH values results are less consistent, particularly after the 1st and 2nd hours (note the sharp rise at pH 6.0 in Tables III and VIII). At the end of 4 hours, however, after the electrolytes have had time to exert their full effect, the figures are reasonably consistent, as indicated in Figs. 1 and 2.

At pH 6.0, the Ringer-Locke solution is most favorable to viability, followed by the distilled water and then by the salt solutions in approximate order of concentration.

The .725 M concentration alone happens to be out of place in both charts, appearing to be more toxic than it should in the case of NaCl and less toxic in the case of $CaCl_2$. Such aberrations must be expected in work of this kind unless a very large series of experiments have been averaged.

The most striking point brought out by these charts is, however, that at pH 8.0 the curve of viability in distilled water does not drop but instead rises slightly while all the salt solutions (except .145 M NaCl) fall sharply; and even the Ringer-Locke solution which was more favorable than water at pH 6.0 falls far below it at pH 8.0. Apparently, in a solution with adjusted reaction and unbuffered (except for the action of the cells themselves), the harmful effect of Na or Ca is accentuated by slight alkalinity and Ringer solution, which is favorable when acid, becomes unfavorable.
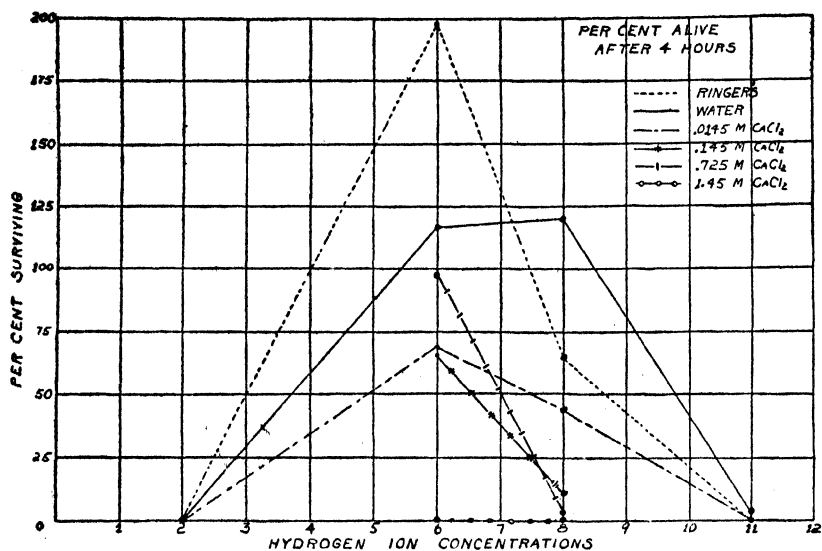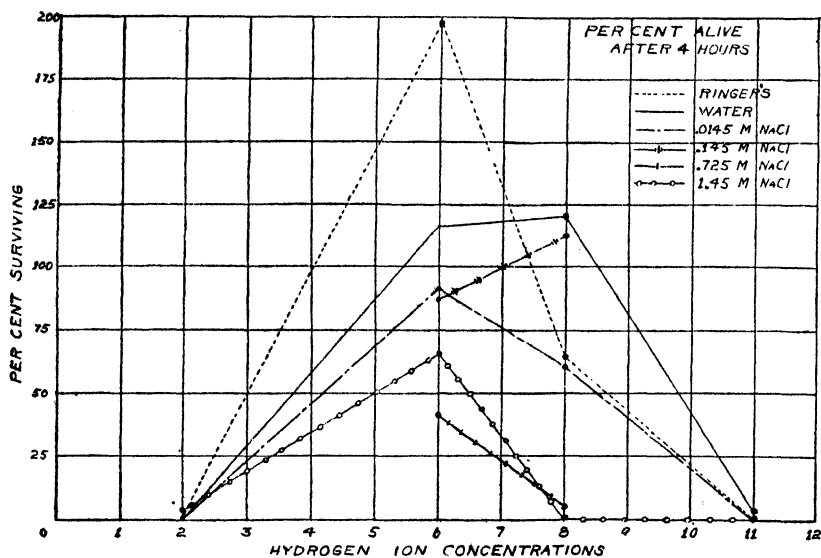
FIG. 1.

FIG. 2

Results in Regard to Electrophoretic Charge.

Our results on migration velocity in the electrical field are presented in Tables XI to XV and in Fig. 3. The figures represent velocity of migration in micra per second in a microscopic cell connected by zinc zinc-sulfate electrodes with a current giving a potential gradient of 12 volts per cm. (see Winslow, Falk, and Caulfield, 1923–24, and Winslow and Fleeson, 1925–26, for description of the technique employed).

A + sign indicates migration toward the cathode; in the absence of any sign, migration is toward the anode.

The time element in these unbuffered solutions proved of no significance, the electrophoretic charge after 1 hour being essentially the same as that observed after 4 hours.

TABLE XI.

Electrophoretic Velocity of Bacterium coli in Distilled Water—Micra per Second.

pH....................	2	6	8	11
Hrs.				
0	+.5	17.3	19.7	8.9
1	+.5	23.6	24.9	13.2
2	+.5	13.8	14.1	13.3
4	0	20.1	20.6	17.0
No. of experiments...	4	4	3	1

TABLE XII.

Electrophoretic Velocity of Bacterium coli in Ringer's Solution—Micra per Second.

pH....................	2	6	8	11
Hrs.				
0	0	4.0	4.0	6.7
1	0	4.1	4.4	7.0
2	0	3.9	4.7	7.2
4	0	3.9	3.9	7.5
No. of experiments...	4	3	2	1

TABLE XIII.

Electrophoretic Velocity of Bacterium coli in Sodium Chloride Solution. 1.45 M. *Micra per Second.*

pH......................	2	6	8	11
Hrs.				
0	0	1.4	4.7	5.0
1	0	1.2	4.3	5.7
2	0	1.6	4.6	5.1
4	0	3.1	6.9	7.0
No. of experiments...	1	1	1	1

TABLE XIV.

Electrophoretic Velocity of Bacterium coli in Sodium Chloride Solution. .0145 M. *Micra per Second.*

pH......................	2	6	8	11
Hrs.				
0	0	9.8	12.1	11.9
1	0	12.2	12.3	11.8
2	0	11.2	12.7	9.8
4	0	12.8	13.5	12.0
No. of experiments...	3	3	2	1

TABLE XV.

Electrophoretic Velocity of Bacterium coli in Calcium Chloride Solution. .0145 M. *Micra per Second.*

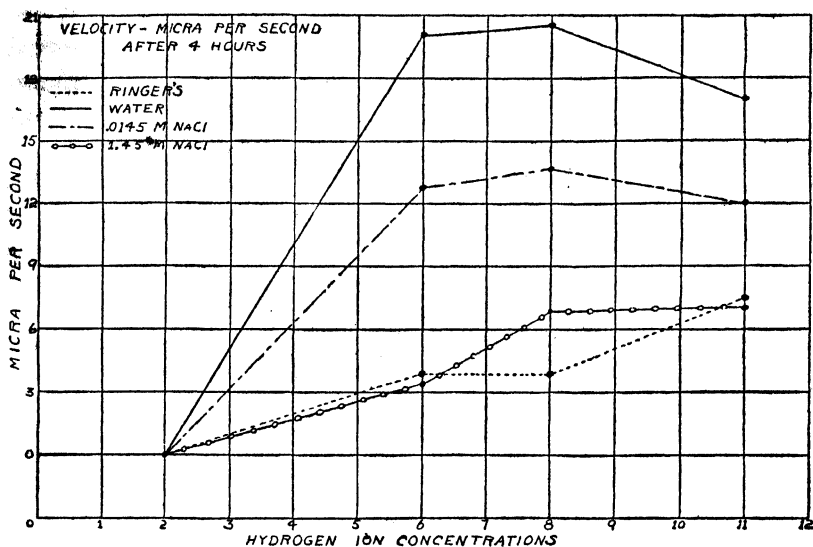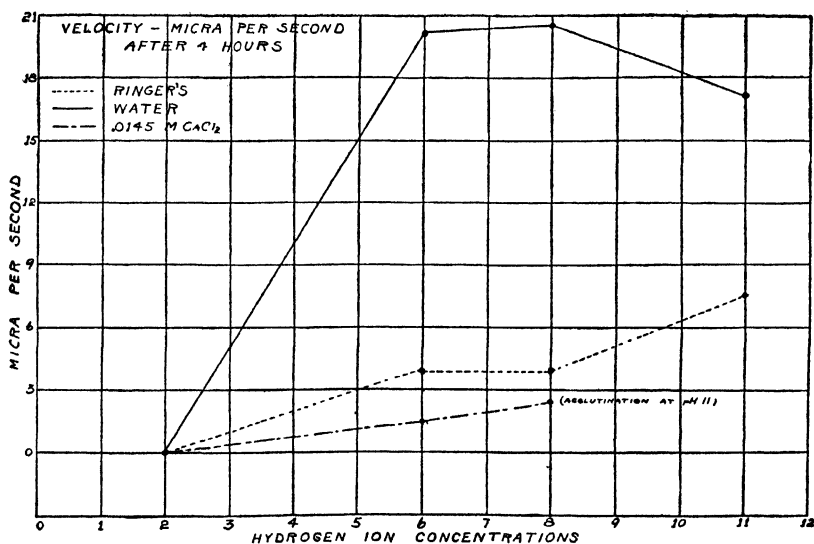| pH......................	2	6	8	11
·Hrs.				
0	0	1.9	1.5	13.6
1	0	1.6	3.3	Aggl.
2	0	1.2	2.8	"
4	0	1.4	2.7	"
No. of experiments...	2	2	1	1

FIG. 3.

FIG. 4.

The highest velocity of migration, as is usual in such experiments, was observed in water at pH 8.0 although water at pH 6.0 gave almost the same value. At pH 2.0 the velocity was greatly reduced, obliterated, or reversed, which might appear to be related to the death of the cells at this reaction. At pH 11.0, however, which is a reaction almost as inimical to viability, the electrophoretic charge falls but slightly. In Ringer solution and the NaCl solutions, indeed, the velocity is greater at pH 11.0 than at pH 8.0. NaCl in .0145 M strength depresses the velocity of the cells only to a moderate degree while Ringer's solution (favorable to viability) depresses it about as does the toxic 1.45 M NaCl and .0145 M CaCl$_2$.

SUMMARY.

1. The strain of *Bacterium coli* used in these experiments multiplies in distilled water at pH 6.0 and pH 8.0 and in Ringer-Locke solution at pH 6.0. Under all the other conditions studied the numbers decrease with the passage of time.

2. The electrophoretic charge of the cells is highest in distilled water at pH 6.0 and pH 8.0. Under all other conditions studied the velocity of migration is decreased, but the decrease is immediate and is not affected by more prolonged exposure.

3. A strongly acid solution (pH 2.0) causes a rapid death of the cells and a sharp decrease in electrophoretic charge, sometimes leading to complete reversal.

4. A strongly alkaline solution (pH 11.0) is almost as toxic as a strongly acid one, although in distilled water the organisms survive fairly well at this reaction. Electrophoretic charge, on the other hand, is only slightly reduced in such an alkaline medium.

5. In distilled water, reactions near the neutral point are about equally favorable to both viability and electrophoretic charge, pH 8.0 showing slightly greater multiplication and a slightly higher charge than pH 11.0. In the presence of salts, however, pH 8.0 is much less favorable to viability and somewhat more favorable to electrophoretic charge than is pH 6.0.

6. Sodium chloride solutions, in the concentrations studied, all proved somewhat toxic and all tended to depress electrophoretic charge. Very marked toxicity was, however, exhibited only in a con-

concentration of .725 M strength or over and at pH 8.0, while electrophoretic migration velocity was only slightly decreased at a concentration of .0145 M strength.

7. Calcium chloride was more toxic than NaCl, showing very marked effects in .145 M strength at pH 8.0 and in 1.45 M strength at pH 6.0. It greatly depressed electrophoretic charge even in .0145 M concentration.

8. Ringer-Locke solution proved markedly stimulating to the growth of the bacteria at pH 6.0 while at pH 8.0 it was somewhat toxic, though less so than the solutions of pure salts. It depressed migration velocity at all pH values, being more effective than NaCl in this respect, but less effective than $CaCl_2$.

9. It would appear from these experiments that a balanced salt solution (Ringer-Locke's) may be distinctly favorable to bacterial viability in water at an optimum reaction while distinctly unfavorable in a slightly more alkaline solution.

10. Finally, while there is a certain parallelism between the influence of electrolytes upon viability and upon electrophoretic charge, the parallelism is not a close one and the two effects seem on the whole to follow entirely different laws.

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THE ELECTRIC CAPACITY OF SUSPENSIONS WITH SPECIAL REFERENCE TO BLOOD.*

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In recent papers (1–4) and in others which will follow, certain theoretical and experimental investigations on the capacity of disperse systems are described, a short summary of which is here given.

THEORY.

We define the specific electric capacity of a suspension as that capacity which, combined in parallel with a certain resistance, electrically balances 1 cm. cube of the suspension. This capacity is rather large for most biological systems, being a measure of that long recognized effect usually called the electrical polarization of the tissue. In general the capacity may be due to one or both of two different causes: (1), a polarization at the interphases of the suspension, and (2), the static capacity of the thin membranes which often are situated at the interphases, such for example, as the cell membranes for the case of biological cells and the adsorption films for the case of protected metallic colloids.

As will be shown in the present paper, the capacity of blood, which is of the order of a few hundred micromicrofarads per 1 cm. cube, is probably due to the second of the above causes. An interesting application of the measurement of the capacity for such a case consists in the calculation of the thickness of the membrane, on which the static capacity depends, a method which probably will be found useful also in investigations of certain non-biological disperse systems, such as protected graphite suspensions which contain well conducting particles surrounded by thin poorly conducting films. The fact that in any

case the capacity of a disperse system is determined by the state of the interphases, makes it probable that it be an important characteristic of the colloidal properties of the system (5); especially for the case of a biological system.

A problem which immediately presents itself when investigating the capacity of suspensions, is the calculation of the capacity per sq. cm. of the interphase in terms of the capacity of the suspension, the volume concentration of the suspended phase, and the geometrical constants of the single suspended particle. For certain important cases this problem has been solved in the papers already referred to. In the present paper we shall only consider the case defined by the requirement that the capacity of the suspension is due either to a static capacity as the result of the existence of a poorly conducting interphasial membrane; or to a polarization, the resistance of which is small as compared to the impedance of its capacity. The frequency is furthermore supposed to be so low that the impedance of the interphasial capacity is high as compared with the interior resistance of the single particle.

For many cases the average particle of the suspension can be considered to be equivalent to a certain spheroid. We have considered such a suspension in a previous paper (3), in which the following formula was derived:

$$C = C_o \cdot \alpha \cdot q\left(1 - \frac{r_1}{r}\right) = C_{100}\left(1 - \frac{r_1}{r}\right) \qquad (1)$$

or

$$C_o = \frac{C}{\alpha \cdot q\left(1 - \frac{r_1}{r}\right)} = \frac{C_{100}}{\alpha \cdot q}, \qquad (2)$$

where C_o is the capacity per sq. cm. of spheroid surface; C, the specific capacity of the suspension; $2q$, the major axis of a spheroid; and r and r_1, the specific resistances of the suspension and of the suspending liquid respectively. C_{100} is the specific capacity of the 100 per cent concentrated suspension.

α depends on the geometrical constants of a spheroid, but is independent of the volume concentration of the suspension. The value of α is given in the paper referred to above and it is tabulated in Table I.

The influence of the volume concentration is expressed solely through the factor $\left(1 - \frac{r_1}{r}\right)$. It may be noted that this part of the formula $\left[C = C_{100}\left(1 - \frac{r_1}{r}\right)\right]$, is of a very general character, being practically independent of the form of the single suspended particle.

TABLE I.

$\frac{b}{a}$	α	$\frac{a}{b}$	α
1	1.50	1	1.50
2	1.30	2	1.03
3	1.27	3	.94
4	1.28	4	.94
∞	1.65	∞	$.118 \cdot \frac{a}{b}$

Experimental Method.

This formula was tested by measurements of the capacity of suspensions of red corpuscles of varying volume concentration. The capacity is of the order of a few hundred micromicrofarads, in parallel to a resistance of a few hundred ohms per 1 cc. of normal blood. It was measured with a specially designed Wheatstone bridge[1] over a range of frequencies from 800 to $4\frac{1}{2}$ million cycles. The sensitivity of the bridge is such that a capacity in parallel to 100 ohms at the lowest frequency can be measured with an accuracy of a few micromicrofarads. Two arms of the bridge (Figs. 1 and 2) contain a Kohlrausch slide-wire which is always used near its middle point; the third arm contains a decade resistance box R_l (General Radio Company, $\frac{1}{10}$, 1, 10, and 100 ohms decades) with a condenser C_l in parallel, and the fourth arm contains the electrolytic cell with a variable condenser C_r (General Radio Company Standard Precision Condenser) in parallel. By means of a switch S the electrolytic cell can be replaced by a decade resistance box R_r similar to R_l. The coils in the resistance boxes are wound by the

[1] This bridge was constructed with the assistance of Mr. I. E. Beasley of this laboratory.

Bridge is connected to shield here

C_2

H Audion generator

Decade condenser C_r

To std. air cond. Air bath

(2)
(1)
(3)

(1) electrolytic cell
(2) thermometer
(3) mercury bath

Hundreds
Tens
Units
Tenths
$R_{(r)}$

S

C_s

Standard air condenser

Hundreds
Tens
Units
Tenths
$R_{(l)}$

Kohlrausch slide-wire

C_0

To detector and three-stage amplifier

Decade condenser C_l

1 meter

G Audion generator 800 to 4½·10⁶ cycles

C_1

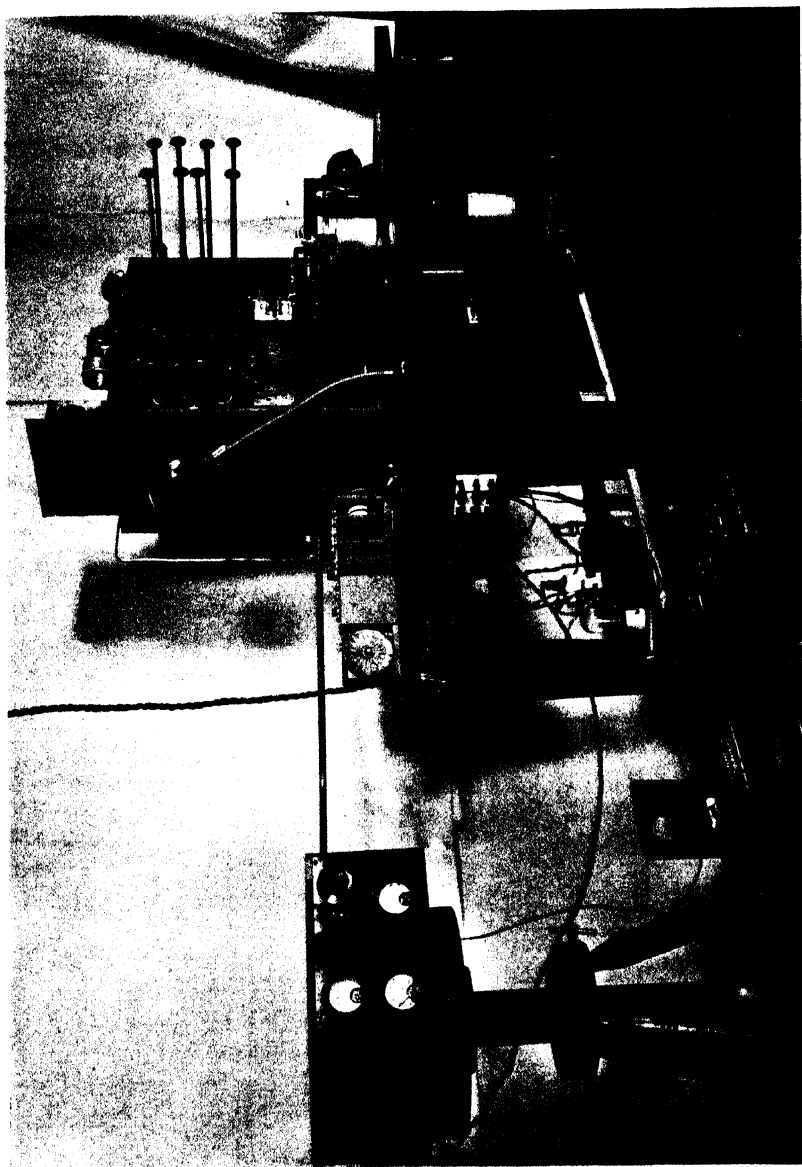Fig. 1. Diagram of high frequency bridge for measuring electric resistance and capacity.

Fig. 2. Photograph of high frequency Wheatstone bridge for measuring electric resistance and capacity.

Ayrton-Perry method and their effective inductances are rather low.
The current to the bridge is delivered by an audion oscillator G
and the heterodyne method of detection is employed, the beat note
being produced by the audion oscillator H; three-stage amplification
is employed. The bridge is connected to the generating and hetero-
dyne oscillators and to the detector tube by the very loose inductive
couplings c_1, c_2, and c_3. All parts of the bridge are carefully shielded,
the bridge being connected to the shield at c_2. The oscillator is
placed at a distance of about 1 meter from the bridge in order to
reduce the direct coupling between them. The electrolytic cell has
the form of an hour-glass with large platinized platinum electrodes
sealed into the glass at the ends; it is designed to have the lowest
possible amount of polarization at the electrodes. Effective stirring,
which is essential, is accomplished by gently blowing through two
glass tubes which are sealed to the top of the cell. (A picture of the
cell is given in Fig. 2 of the following paper (7).)

A substitutional method is employed, in which the blood in the
last analysis is compared with a diluted serum of the same specific
resistance as the blood, the blood and the serum being placed con-
secutively in the same electrolytic cell. The abstract of the protocol
given in Table II will explain the procedure. The cell is filled with
the blood and a balance is first obtained with the cell in (switch S
down); the settings of the left resistance box R_l', of the standard
condenser C_r', and of the slide-wire are noted; a balance is there-
after obtained with the right resistance box R_r in (switch S up)
and the settings R_r'' and C_r'' noted; if the setting of the slide-wire
had to be changed in the last measurement, a change of $\frac{1}{10}$ ohm is
made in R_r and the slide-wire and standard condenser are changed
until equilibrium is again established. R_r'' is the resistance of the
suspension, while $C_r''-C_r'$ represents an uncorrected value for the
capacity. This value is first corrected for the inductance of the coils
used in the resistance box R_r, and for the difference between the in-
ductances of the leads which connect the cell and R_r respectively to
the bridge; this correction for our case is $\frac{L}{R_r''^2}$ farad when this total
inductance is L henry. A small correction is thereafter introduced

TABLE II.

Abstract of Protocol.

Blood (11.1 per cent).

No. of experiment.	IV_1		IV_2	IV_3	IV_4	Diluted serum.			
R'_t (ohms)	93.3		93.0	93.2	93.6	93.1		187.8	189.0
C'_t (arbitrary units)	6.47		6.43	6.61		8.08		8.18	
Setting of slide-wire	−1		−7	−7	−7	−1		−7	−8
R''_t (ohms)	93.9	93.8	93.5	93.7	93.6	93.7	93.8	189.1	189.1
C''_t (arbitrary units)	10.05	10.43	10.10	10.26	10.68	10.72	10.40	9.27	9.34
Setting of slide-wire	−14½	−7	−7	−2½	−24½	0	−17½	−1	−8
Temperature	23.30°		23.12°	23.20°	23.20°	22.40°		22.40°	
$C''_t - C'_t$ (arbitrary units)	3.58	3.95	3.67	3.65	4.07	2.64	2.32	1.09	1.16
$C''_t - C'_t$ (m.m.f.)	217	240	223	222	247	160	141	66	70.
Inductance of coils of resistance box. $\left(10^{-6}\text{ henry.}\right)$ $\left\{ L_{100} \right.$	8760		8760	8760	8760	8760		2270	
L_{10}	1970		1970	1970	1970	1970		7950	
L_{1}	1960		1130	1650	1470	1650		5120	
$\left. L_{.1} \right.$	1820	1820		1650	1650		1820	370	
Difference of inductance of leads respectively to box and to cell	−900				−900	−900		−900	
Total inductance (10^{-9} henry)	11,790	11,650	10,960	11,480	11,300	11,480	11,650	14,810	14,440
Equivalent capacity $\left[=\dfrac{\text{total inductance}\cdot 10^2}{R''^2_t}\right]$ m.m.f.	134	123	126	130	129	130	132	41.	40.
Capacity corrected for inductance	83	108	98	92	118	30	9.	25	30
Capacity corrected for difference in slide-wire setting	101		98	97		29		26	
Capacity corrected for static capacity of electrolytic cell	95		92	91		21	21	18	
Capacity for cell filled with serum	21		21	21					
Capacity of blood	74		71	70					

for the static capacity of the electrolytic cell, which for low frequencies

is $\dfrac{7.2}{\left(\dfrac{r}{r_1}\right)c}$ micromicrofarads in which r and r_1 are specific resistances of

suspension and intercellular liquid respectively and c is the constant of the electrolytic cell $\left(\dfrac{\text{resistance}}{\text{specific resistance}}\right)$.

The value thus corrected is still faulty on account of the difference in static couplings between the electrolytic cell and other parts of the bridge on one side, and on the other side between the resistance box R_r and other parts of the bridge. The corresponding correction, called the zero capacity, depends on the resistance; it is obtained by making a series of similar measurements with the cell filled with various dilutions of serum. The values of the zero capacity thus obtained are plotted against the resistances. The final value for the capacity of the blood is obtained by subtracting the zero capacity.

The procedure described above would not have given a correct elimination of a polarization at the electrodes of the electrolytic cell if such an effect had been present to any appreciable amount within the experimental range of frequencies. The frequency at which the polarization becomes appreciable is easily determined by measuring the serum at decreasing frequencies; the setting of the standard condenser remaining practically constant until the critical frequency is reached, when an abrupt change takes place.

A confirmation of the accuracy of the method described was obtained by making measurements on cream in which case the resulting capacity is zero. The fact that the value of the capacity of a corpuscle suspension is found to be independent of the form of the electrolytic cell and of the frequency serves as a further confirmation. (The capacity is found to be independent of the frequency between 3600 and 87,000 cycles per second; for higher frequencies the capacity decreases because of the fact that the impedance due to the static capacity of the corpuscle membrane becomes comparable with the impedance of the corpuscle interior.)

Measurements of Suspensions of Red Corpuscles.

In Tables III and IV is given a complete series of measurements with suspensions of the red corpuscles of a dog. This series is typical of several others, in which, besides the blood of a dog, the blood of rabbits has also been used. C_{100} (the capacity for a 100 per cent solution) is calculated by formula (1) and except perhaps for the very

TABLE III.

Capacity of Suspensions of Red Corpuscles in Own Serum.

No. of experiment.	Volume concentration calculated from resistance.	Resistance (r) (ohms).	Capacity (C) m.m.f.	Capacity (C_{100}) for 100 per cent concentration (calculated).	Date, Mar. 24, 1925. Origin.
	per cent				
I	43.9	188.7	232	388	Original blood.
II	30.8	140.1	172	374	From original blood by dilution.
III	20.6	113.7	126	378	From 30.8 per cent suspension by dilution.
IV	11.1	94.0	72	371	From 20.6 per cent suspension by dilution.
V	10.6	93.4	74	391	From original blood by dilution.
VI	42.8	185.3	221	374	Original blood.

Average: 380 m.m.f.
±2 per cent.

Defibrinated blood of Dog 1 was diluted with own serum.

Resistance (r_1) of serum: 75.8 ohms. Temperature: 23.10°C. Constant of electrolytic cell: .98. Specific capacity for 100 per cent concentration: 372 m.m.f. Capacity per sq. cm. of membrane: $C_o = .81$ m.f.

highest volume concentrations is constant within the experimental error, which is a few per cent.

The volume concentration is calculated from the resistance by a formula recently given (6) in which $\dfrac{a}{b}$ (ratio of thickness to diameter of corpuscle) is taken as equal to $\frac{1}{4}$.

The average value of C_{100} for the blood used in Table I is equal to 380 m.m.f., the constant of the electrolytic cell being .98. The corresponding specific capacity is obtained by multiplying the average value of C_{100} with the constant of the electrolytic cell and therefore is equal to $380 \cdot 98 = 372$ m.m.f. This value would be too small $\left(\text{with a factor of the order of } \left(\dfrac{r_1}{r_1 + r_2}\right)^2\right)$ if the resistance of the

TABLE IV.

Capacity of Suspensions of Red Corpuscles in Own Serum.

No. of experiment.	Volume concentration calculated from resistance.	Resistance (r) (ohms).	Capacity (C) m.m.f.	Capacity (C_{100}) for 100 per cent concentration (calculated).	Origin.
	per cent				
I	83.9	931.	374	411	Concentrated by centrifugation from original blood.
II	21.0	126.9	129	385	From 83.9 per cent suspension by dilution.
III	72.0	498.	343	411	From 83.9 per cent and 21. per cent suspensions.
IV	47.5	230.2	237	374	From 72.0 per cent suspension by dilution.
V	60.2	329.	286	385	From 83.9 per cent and 47.5 per cent suspensions.

Blood of dog. Frequency: 87,000 cycles per sec. Date, Mar. 23, 1925.

Defibrinated blood of Dog 1 was concentrated by centrifugation, and the concentrated suspension was diluted with serum.

Resistance (r_1) of serum: 84.25 ohms. Temperature: 18.95°C. Constant of electrolytic cell: $c = .98$.

[2] Polarization by alternating current has been hitherto mainly investigated at metal electrodes. For small current densities it is equivalent to a capacity in series with a resistance. Only a few simple cases have been adequately investigated theoretically and experimentally and our knowledge of the subject is still very limited and is restricted mainly to the very low frequencies (under 1000 cycles). One important type of polarization is caused by the change in concentration at the electrode surface due to the electric current, of one or

corpuscle membrane (r_1) were appreciable compared with the resistance of the mass of the inter- and intracellular liquid (r_2) which is in series with the membrane (compare Fig. 1, diagrams (a) and (c) of the following paper (7)). A lower value for the resistance of the membrane may be derived from the maximum value of the resistance of the concentrated corpuscles of the blood of a dog which we can obtain by centrifuging the blood for a long time at the highest possible speed. It is about 60 times the value for the serum. According to observations of the resistance of blood at very high frequencies (7) the specific resistance of the interior of the corpuscle is about 3.5 times the specific resistance of the serum, so $\frac{r_1}{r_2}$ is at least equal to $\frac{60}{3.5} = 17$; consequently the factor by which C_o may be too small, is of the order of $\left(\frac{18}{17}\right)^2 = 1.12$, which is negligible compared with what may be due to other sources of uncertainty.

In order to calculate C_o, the capacity per sq. cm. of surface, we use

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several substances on which the electrode potential depends, such as the cation for a metal in a solution of one of its salts and hydrogen gas for platinum in an acid. This concentration variation produces a change of the normal electrode potential, which is directed against the electric current and in effect is equivalent to the presence of a capacity at the electrode surface. The variation of this capacity with the frequency depends on the process by which the substances in question are removed from the geometrical surface of the electrode. In one important case, which has been discussed theoretically by Warburg (8), the removal is solely due to ordinary diffusion; here the maximum change of concentration during one cycle varies directly and therefore the polarization capacity varies inversely as the square root of the frequency. This case is realized experimentally with a few non-polarizable electrodes, such as mercury in a solution of mercuric sulfate in sulfuric acid or silver in an acid solution of silver nitrate (9). The capacity is here proportional to the concentration of cation and for a 1/1000 N solution and 1000 cycles is of the order of a few hundred microfarads. It may also be noted that according to investigations by Gildemeister (10) the polarization in frog skin follows Warburg's theory for frequencies between 400 and 90,000 cycles.

Metals in solutions which do not contain the metal ion or contain it in very low concentration usually have a low polarization capacity, which varies more slowly than with the inverse square root of the frequency, being independent of the frequency (11) in certain cases within the limited frequency ranges investigated. The capacity is of the order of 10 m.f. per sq. cm., being not very

$\frac{a}{b} = \frac{1}{4}$, which value was found to secure best agreement between the volume concentration of the blood of a dog as directly observed by Stewart and as calculated by a formula which has been recently derived (6). α (Table I) accordingly equals 1.28.

For the diameter of a corpuscle we use $2q = 7.2 \cdot 10^{-4}$ cm. Consequently by formula (2): $C_0 = \dfrac{372}{3.6 \cdot 10^{-4} \cdot 1.28 \cdot 10^{-6}}$ microfarad = .81 microfarad.

The experiments in Tables II and III were made with a frequency of 87,000 cycles per second; it is found, however, that the capacity is independent of the frequency, when this is lower than about 100,000 cycles per second. The lowest frequency which we have used has been 3600 cycles. The accuracy of the determinations over this range of frequencies is within a few m.m.f. For frequencies over 100,000 cycles per second the capacity begins to decrease. For frequencies up to $4\frac{1}{2}$ million cycles, however, as has been shown elsewhere (7), this decrease is solely—or very nearly so—due to the im-

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different for different metals and solutions; it is usually very inconstant and unreproducible, as would be expected since the same is true for the potential. A theory of Krüger (12) explains polarizations of this type by taking into account the absorption or liberation of metal ions by the electrode, which takes place when the electrode potential changes. The concentration variation is thereby reduced and as will be easily seen, this effect must approximately be equivalent to a constant capacity of the order of the static capacity corresponding to the thickness of the dipole layer, by which the electrode potential is produced. By this effect the polarization capacity of a non-polarizable electrode should therefore become constant and equal to about 10 m.f. when the frequency is very high or the concentration of the metal ion very low. A series of experiments has been reported by Krüger which shows the continuous change from the inverse square root law to constancy for mercury in solutions of decreasing strength of mercuric sulfate. However, it is to be noted that in certain cases other effects are probably also of importance in producing constant capacities, as for instance the presence of non-conducting oxidation films (monomolecular) on the surface of the electrode (compare (13)). Especially extended investigations have been made with platinum, when the polarization under different conditions of the platinum (its content of occluded hydrogen) may depend on the variations of the hydrogen ion concentration or on the variations of the pressure of the hydrogen gas at the surface of the platinum. In certain experiments (14) the square root

pedance of the inter- and the intracellular liquid, with which the capacity of the corpuscular membrane is in series. We may, therefore, conclude that the capacity per sq. cm. of corpuscle surface is independent of the frequency between 3600 and $4\frac{1}{2}$ million cycles.

It is also found that the capacity is not changed, when the corpuscles from defibrinated blood are transferred to Ringer's solution or to an isotonic sugar solution. On the basis of these experimental data and our present knowledge of polarization[2] it seems unlikely that the observed capacity should be due to a polarization at the surface of the red corpuscles rather than to the static capacity of a membrane surrounding the corpuscle. Furthermore (compare equation (2) of the following paper (7)), even if the capacity were due to a constant polarization at the corpuscle surface, the observed capacity would be independent of the frequency only when the resistance of the corpuscle membrane is low as compared with the impedance of the capacity over the whole range of experimental frequencies. Different considerations seem to indicate that this is not the case; however, knowing as little as we do about the nature of the membrane, it is impossible to draw definite conclusions.

In the following we shall calculate how thick the membrane must be if the observed capacity is due solely to the static capacity of the membrane. Assuming a value of 3 for the dielectric constant of the membrane (a value which is, of course, rather uncertain, especially since the mem-

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law is found to hold, in others we have the constant capacity or intermediate cases (11). Finally for the sake of completeness, we may mention that cases are also found in which the capacity varies faster than with the inverse square root. According to Krüger (12) this condition is realized when there are present in the solution complex compounds which are slightly dissociated into compounds of which one is the substance on which the polarization depends. In this case the variation of concentration of this substance is counteracted by the dissociation or production of the complex compound and the capacity therefore is higher. The calculation shows that in the ideal case it varies inversely as the first power of the frequency. Krüger has realized this condition experimentally with mercury in solutions of complex mercury salts (for instance in N KCNS $+ \dfrac{1}{100}$ M HgCNS); it is also approximately realized for the case of palladium black saturated with hydrogen gas (15), in which the hydrogen is present mainly in the molecular form, dissociating slightly into atomic hydrogen, on which the electrode potential depends.

brane appears to be monomolecular), and by means of the formula
$C_0 = \dfrac{3}{4\pi \cdot x \cdot 9 \cdot 10^5}$ microfarads, we obtain for x, which represents the
thickness, the value $3.3 \cdot 10^{-7}$ cm. This value corresponds to from 20 to
30 carbon atoms, if we assume that the distance between two neighboring carbon atoms of an organic molecule is 1 to $1\frac{1}{2} \cdot 10^{-8}$ cm., a
value which Langmuir (16), for instance, derived by his investigations
of the spreading of different fatty acids on the surface of water. It is
evident, therefore, that our value for the thickness corresponds to a
monomolecular membrane.

At present nothing is definitely known concerning the nature of
the membrane, and therefore it is impossible to draw conclusions regarding the probability that this is the correct value for the thickness.
We may only note that this value for the length of a single molecule of
lipins (such as lecithin, cholesterol, etc.) such as chemically may be
related to the membrane substance, is about what one would expect
from what is known of their chemical formulæ. In this connection
we may also mention that du Noüy (17) derives a value of $3.8 \cdot 10^{-7}$ cm.
as the diameter of a serum protein molecule by investigating the surface tension of diluted serum protein solutions.

<div align="center">SUMMARY.</div>

1. The specific capacity of a suspension is that capacity which,
combined in parallel with a certain resistance, electrically balances 1
cm. cube of the suspension.

2. The following formula holds for the specific capacity of a suspension of spheroids, each of which is composed of a well conducting
interior surrounded by a thin membrane of a comparatively high
resistance:

$$C = C_o \, \alpha \, q \left(1 - \frac{r_1}{r}\right) \tag{1a}$$

C, specific capacity of suspension; C_o, static capacity of one sq. cm.
of membrane; r, r_1 specific resistances respectively of suspension and
of suspending liquid; $2\,q$ major axis of spheroid, α constant tabulated
in Table I.

3. The following formula holds practically for any suspension whatever the form of the suspended particle.

$$C = C_{100} \left(1 - \frac{r_1}{r} \right) \tag{1b}$$

C_{100} being the specific capacity of a suspension with a concentration of 100 per cent.

Formulæ (1a) and (1b) hold only for the case, when the frequency is so low, that the impedance of the static capacity of the membrane around a single particle is high as compared with the resistance of the interior of the particle. The formulæ hold also for a suspension of homogeneous particles, when polarization takes place at the surface of each particle, provided the polarization resistance is low as compared with the impedance of the polarization capacity.

4. A description is given of a method for measuring the capacity of a suspension at frequencies between 800 and $4\frac{1}{2}$ million cycles. By means of a specially designed bridge, a substitution method is employed, by which in the last analysis the suspension is compared with the suspending liquid which is so diluted as to have the same specific resistance as the suspension, consecutive measurements being made in the same electrolytic cell.

5. Formula (1b) is verified by measurements of the capacity of suspensions of varying volume concentrations of the red corpuscles of a dog.

6. By means of the above measurements, the value of C_o is calculated by equation (1a).

7. It is found that C_o is independent of the frequency up to $4\frac{1}{2}$ million cycles and that it is also independent of the suspending liquid. These results furnish considerable evidence of the validity of the theory, that C_o represents the static capacity of a corpuscle membrane.

8. On this assumption and using a probable value for the dielectric constant of the membrane, the thickness of the membrane is calculated to be *3.3·10⁻⁷ cm.*

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THE ELECTRIC RESISTANCE AND CAPACITY OF BLOOD FOR FREQUENCIES BETWEEN 800 AND 4½ MILLION CYCLES.

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GENERAL.

In this report a series of measurements is presented upon the electric resistance and capacity of blood (as defined in the preceding paper) at from 800 up to 4½ million cycles. For low frequencies the resistance and capacity are found to be independent of the frequency of the electric current; above a certain frequency (ω), however, they both begin to decrease. It is found that the experimental values ($R(\omega)$; $C(\omega)$) approximately correspond to the two simple equations:

$$\frac{1}{R(\omega)} = \frac{1}{R_o} + \frac{1}{R_i}\frac{C_o^2\omega^2 R_i^2}{1 + C_o^2\omega^2 R_i^2} \tag{1}$$

$$C(\omega) = \frac{C_o}{1 + C_o^2\omega^2 R_i^2} \cdot \tag{2}$$

These equations are derived by considering the blood as equivalent to the system shown in the diagram (*a*) of Fig. 1 $\left(\dfrac{1}{R_o} + \dfrac{1}{R_i}\right)^{-1}$ representing the resistance of the blood at infinite frequency.

The diagram expresses the fact that the current which passes through the capacity of the membrane has to go through a certain resistance (R), composed of the resistance of the interior of the corpuscle and of the resistance of a certain mass of the intercellular liquid which lies in front of and behind the corpuscle.
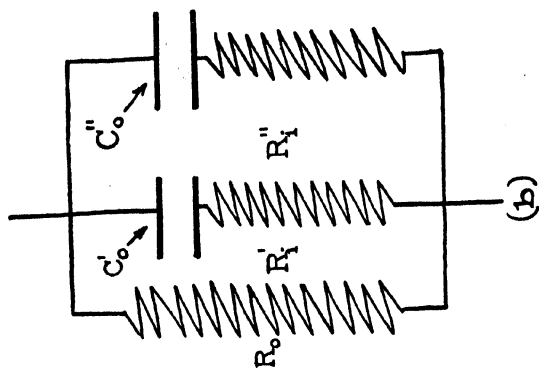
Diagram (*a*) is of course only a rough representation of actual facts, and equations (1) and (2) are accordingly only approximations. We expect in another publication to treat the problem by a more exact
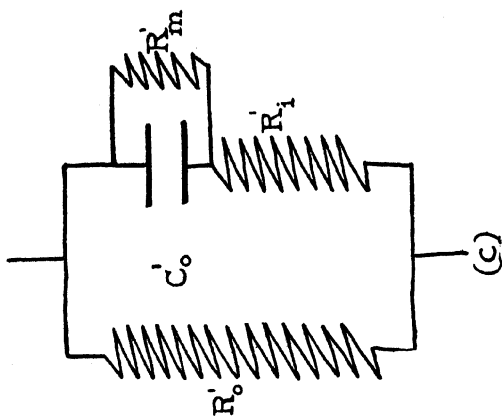
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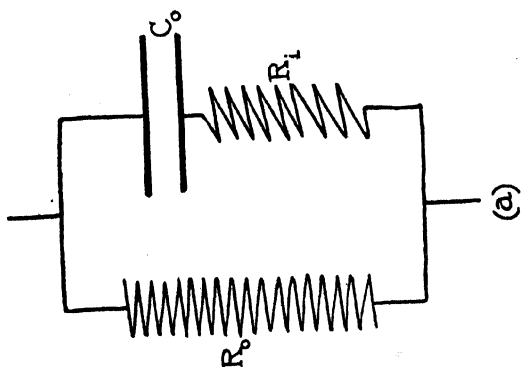
$$R(\omega) = \left[\frac{1}{R_o} + \frac{1}{R_i} \frac{C_o^2 \omega^2 R_i^2}{1 + C_o^2 \omega^2 R_i^2} \right]^{-1} \qquad (1)$$

$$C(\omega) = \frac{C_o}{1 + C_o^2 \omega^2 R_i^2} \qquad (2)$$

$$R(\omega) = \left[\frac{1}{R_o} + \frac{1}{R_i'} \frac{C_o'^2 \omega^2 R_i'^2}{1 + C_o'^2 \omega^2 R_i'^2} + \frac{1}{R_i''} \frac{C_o''^2 \omega^2 R_i''^2}{1 + C_o''^2 \omega^2 R_i''^2} \right]^{-1} \qquad (3)$$

$$C(\omega) = \frac{C_o'}{1 + C_o'^2 \omega^2 R_i'^2} + \frac{C_o''}{1 + C_o''^2 \omega^2 R_i''^2} \qquad (4)$$

$$C_o = C_o' \left[\frac{R_m'}{R_i' + R_m'} \right]^2$$

$$R_i = \frac{(R_m' + R_i') R_i'}{R_m'}$$

$$R_o = \left[\frac{1}{R_o'} + \frac{1}{R_m' + R_i'} \right]^{-1}$$

FIG. 1. Electrical diagrams of blood.

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method. It would undoubtedly be more logical to consider the capacity as composed of different parts, each of which has a different value for $C_o \cdot R_i$. For the case in which the capacity is composed of two parts, C_o' and C_o'' as shown in diagram (b) of Fig. 1, we obtain the following substitutes for the equations (1) and (2)

$$\frac{1}{R(\omega)} = \frac{1}{R_o} + \frac{1}{R_i'}\frac{(C_o'\omega R_i')^2}{1 + (C_o'\omega R_i')^2} + \frac{1}{R_i''}\frac{(C_o''\omega R_i'')^2}{1 + (C_o''\omega R_i'')^2} \tag{3}$$

$$C(\omega) = \frac{C_o'}{1 + (C_o'\omega R_i')^2} + \frac{C_o''}{1 + (C_o''\omega R_i'')^2} \tag{4}$$

We would certainly expect that equations of this form, (3) and (4), rather than of the simple form, (1) and (2), would have to be used for blood since here generally $C_o R_i$ depends on the orientation of the single corpuscle. The application of (3) and (4) rather than of (1) and (2) would of course be imperative if there existed a considerable number of corpuscles in the blood essentially different from the majority, and also if there should exist a region in each corpuscle which, as regards $C_o R_i$, is very different from the rest of the corpuscle.

It may be well to point out that equations of the form (1) and (2) (or (3) and (4)) will also hold for the case in which the cell membrane is conducting; this condition is shown in diagram (c) of Fig. 1: the corresponding formulæ for resistance and capacity are obtained from (1) and (2) by using:

$$C_o = C_o' \left(\frac{R_m'}{R_i' + R_m'}\right)^2$$

$$R_i = \frac{R_m'}{R_m' + R_i'} R_i'$$

and

$$\frac{1}{R_o} = \frac{1}{R_o'} + \frac{1}{R_m' + R_i'}$$

In order to simplify the problem, we have assumed in deriving the foregoing formulæ that the capacity of the blood is due solely to the static capacity of the membrane around the red corpuscle. This assumption is strongly supported by our experimental results; however, the formulæ will hold equally well for the case in which the

capacity is due to polarization at the interphases, when C_o and R_i are considered to be dependent on the frequency.

Calculation of Specific Resistance of Corpuscle Interior.

By the application of formula (1) (or (3)) to our experimental data, the resistance of the blood for infinite frequency can be extrapolated with considerable accuracy. When $\omega = \infty$ formula (1) reads:

$$\frac{1}{R(\infty)} = \frac{1}{R_o} + \frac{1}{R_i}$$

In an earlier paper (1) the following formula was derived for the case of a suspension of homogeneous spheroids:

$$\frac{1 - \dfrac{r}{r_1}}{1 - \dfrac{r}{r_2}} \left(\frac{r_1}{r_2} - 1 \right) = \beta \frac{\rho}{1 - \rho} \tag{5}$$

in which r, r_1, and r_2 are the specific resistances of the suspension and of the suspending and the suspended phases respectively, ρ is the volume concentration of the suspension, and β is a constant which depends on the ratio $\left(\dfrac{a}{b} \right)$ of the thickness to the width of the spheroid and on $\dfrac{r_1}{r_2}$, and which is represented graphically in the paper referred to above. By means of this equation, using for r the value $(R(\infty))$ of the resistance of the blood at infinite frequency, we can calculate r_2, which is the specific resistance of the interior of a red corpuscle.

HISTORICAL NOTE.

The electric resistance of blood for currents of very high frequencies has already been investigated by R. Höber (2). From his experimental results he drew the conclusion that the conductivity of the interior of a red corpuscle lies between that of a 0.1 and a 0.4 per cent NaCl solution, the limits being determined by the accuracy of his experimental methods. (We may mention that the present measurements lead to a value of about 0.17 per cent.)

Recently M. Philippson (3) has made a single series of determina-

tions of the impedance of a suspension of the red corpuscles of a horse for frequencies between 1000 and $3\frac{1}{2}$ million cycles. Philippson's method consisted in a simultaneous measurement of the current and of the potential across the electrolytic cell by means of a tube voltmeter. The suspension was obtained by a 1 hour centrifugation. The value of the specific resistance of the interior of a corpuscle (3.15 times the specific resistance of the serum) as calculated from his experimental results, is in fair agreement with our own values for other animals; on account of the prolonged centrifugation before the measurement, the value derived from Philippson's data is probably somewhat lower than the normal value.

<center>EXPERIMENTAL.</center>

The method of measurement has already been described in earlier reports (4) regarding the capacity of blood (see especially the preceding paper), and we shall add here only some later developments and certain details which are of especial importance in measurements at very high frequencies.

Fig. 2 is a diagram of the electrolytic cell used in most of the experiments presented here. Several other types of cell, however, have been used in the course of the investigation for the sake of comparison or of convenience. Different sizes of the cell shown in Fig. 2 hold from 10 to 75 cc. of blood. The cell is so designed as to have a low polarization at the electrodes, which are made of platinum and covered with platinum black and are fused into the glass. The cell is divided into two cup-shaped halves, between which a celluloid diaphragm is inserted. The edge of each half which is in contact with the diaphragm is broadened and ground flat and circular. The diaphragm, which is also circular and of the same size, can thus be easily centered and the whole cell when clamped together is easily made water-tight. We use a set of these diaphragms, in the centers of which are holes of different areas. By means of these diaphragms varying and readily reproducible cell constants can be obtained over a considerable range. For the measurement of blood we usually employ a cell constant of about 1.

Stirring is attained by displacing the suspension back and forth between the two bulbs which are attached to the inlet tubes of the
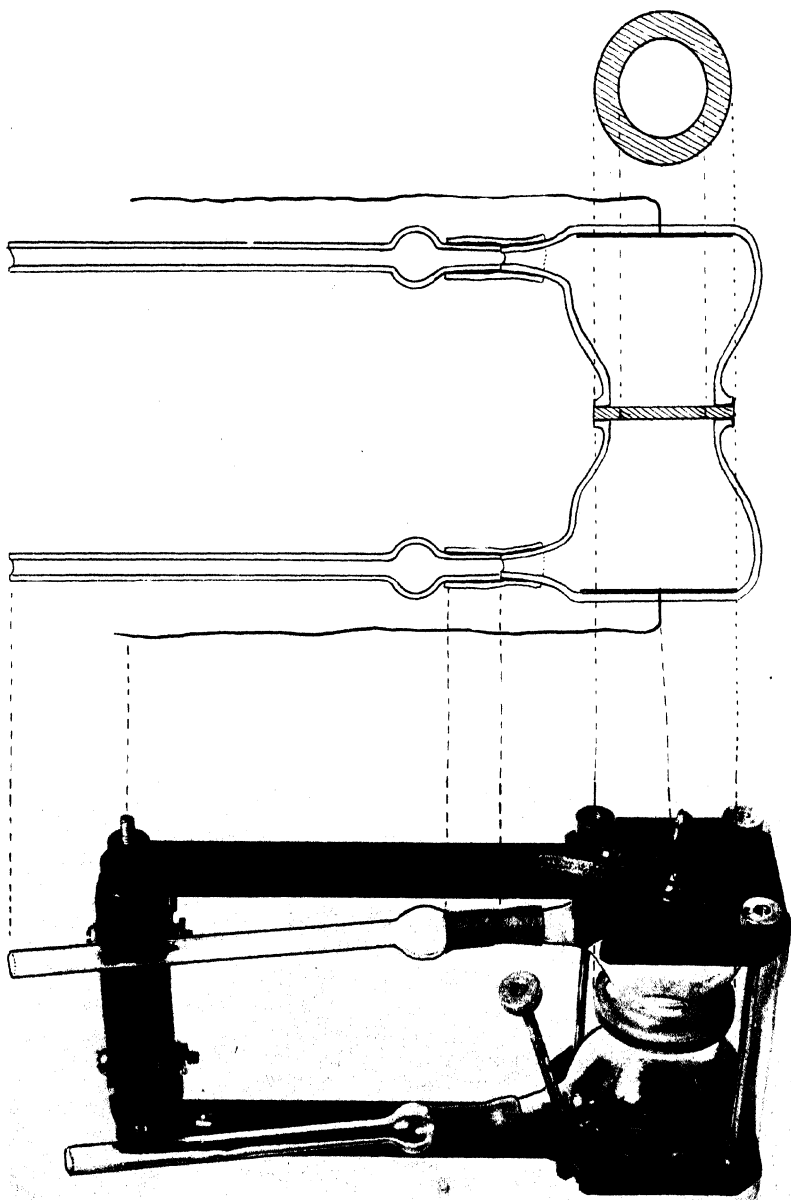
FIG. 2. Conductivity cell for measuring electric resistance and capacity of blood.

cell. The stirring is a very important procedure. As soon as it is stopped, the resistance and capacity usually rise rapidly to new and rather fixed values. This change, which amounts to a few per cent and is accomplished within a minute or less, can hardly be due to an ordinary settling of the corpuscles. We have observed a quite similar effect with cream (5) and we believe that both of these effects are due to the establishment of some kind of an ordered arrangement of the particles of the suspensions, independent of gravity. In this connection it is interesting to note that for certain specimens of blood this effect was much more pronounced than for others; the same was true with cream. In the investigations reported here we always endeavored to measure the resistance and the capacity exactly at the moment when the stirring was stopped, for the reason that in this work we use a formula derived under the assumption that the suspended particles are distributed at random. In our work with cream (5) we found that the formula for this case is exact only when the resistance is taken immediately after stirring and consequently conclude that the assumption of random distribution is only fulfilled at that moment. That the same holds for blood is not certain, of course; in any case the effect is so small that it is of minor importance in the present work.

For the control of the temperature during the measurements the cell is placed in an air bath, at the bottom of which is a container filled with mercury. This container can be raised so that the cell may be immersed in the mercury, thus accelerating the establishment of temperature equilibrium.

For the measurements at the higher frequencies the following point must be noted. When a homogeneous liquid is measured by substituting it in a bridge against the units of a resistance box, it will usually be found that the resistance of the liquid apparently changes with the frequency. For the bridge here used the effect is negligible for frequencies under 1 million cycles and amounts to a few per cent for higher frequencies up to about 3 million cycles. For still higher frequencies it may amount to as much as 10 per cent. This effect is principally due to the difference of the coupling between the cell and the bridge on the one hand and the substituted resistance box and the bridge on the other, and, at the very

highest frequencies, in part to the fact that the resistance of the coils of the resistance box is not independent of the frequency, owing to the presence of an appreciable amount of inductance and distributed capacity in the coils.

The difference between the true resistance and the apparent resistance which depends not only on the frequency but also on the resistance, we shall term the resistance defect. A table of the resistance defects for different resistances and frequencies is made up in the following way. The cell is filled with serum and a series of different diaphragms is placed consecutively in it, the diaphragms being so chosen, that the resistances obtained are distributed regularly over the range inside which our experimental resistances fall. Measurements of resistance and capacity are made at all experimental frequencies for each diaphragm; the difference between the resistance values obtained at such a low frequency that the defect is inappreciable, and at a certain high frequency, gives the resistance defect for that resistance and frequency. By employing the procedure of the preceding paper, these measurements will also give the zero capacity. Practically the same values for the resistance defect and the zero capacity are obtained when the resistance is varied by using different dilutions of the serum, as was done in the preceding paper. The resistance defect and the zero capacity are measured only once for each cell, perhaps with an occasional rechecking; their values are nearly the same for all the cells which we have used.

An abstract of the protocol of measurements is given in Table I.

The method described is not practical at the very highest frequencies (over about 3 million cycles), because at these frequencies the resistance and inductance of the coils of the resistance box change with the frequency. We employ here a strict substitution method. After having finished the measurements on the blood at all the different frequencies, the cell is filled with serum and consecutive measurements are made with different specially chosen diaphragms in the cell. Each diaphragm is so chosen that the resistance of the cell at one of the frequencies in question, will be practically equal to the resistance of the blood at the same frequency. With each of these diaphragms we make a measurement at the high frequency and repeat at a low frequency, the difference between the values of the resistances thus

secured being the resistance defect, which must be added to the observed value for the resistance of the blood at the same frequency. The correct capacity of the blood is the difference between the settings of the right condenser (C_r) for the blood and for the serum respectively.

It may be well to note here, also, that the procedure which we have described does not completely eliminate the influence of polarization at the electrodes of the cell. Therefore it is always necessary to ascertain that the polarization is negligible at all frequencies with which we desire to work. The frequency at which the polarization becomes appreciable is easily found by measuring the serum at decreasing frequencies. The setting of the condenser C_r stays very nearly constant until the critical frequency is reached when an abrupt change begins.

TABLE I.

Capacity and Resistance of Blood of Calf for Frequencies from 87,000 to 4½ Million Cycles.

Jan. 19, 1925.

Cycles per sec. $(\omega/2\pi)$.	Capacity (m.m.f.).	Resistance (ohms).	$\dfrac{146}{1 + (146 \cdot 10^{-12} \cdot \omega \cdot 370)^2}$	$\left[\dfrac{1}{370} \cdot \dfrac{(146 \cdot 10^{-12} \cdot \omega \cdot 370)^2}{1 + (146 \cdot 10^{-12} \cdot \omega \cdot 370)^2} + \dfrac{1}{191}\right]^{-1}$
87,000	146	191	146	191
833,000	130	181	135	183
$1.17 \cdot 10^6$	118	174	126	178
$1.52 \cdot —$	106	168	115	172
$2.04 \cdot —$	90	159	98	163
$3.04 \cdot —$	68	148	71	151
$3.82 \cdot —$	60	144	55	144
$4.52 \cdot —$	39	138	43	140
∞	—	(124) extrapolated.	—	126

Blood of calf, defibrinated, concentrated by centrifugation.

Volume concentration of suspension: **46.0 per cent** $\left[\dfrac{a}{b} = \dfrac{1}{3}\right]$.

Constant of electrolytic cell: .856.

Capacity of 1 cc. of blood for a volume concentration of 100 per cent: $\dfrac{146 \cdot .856}{.60} =$

208 m.m.f.

Temperature: 21.6°C. Specific resistance of serum: **89.4 ohms.**

TABLE I—*Concluded.*

Specific resistance of inside of corpuscles is **3.5 ± 5 per** cent *times specific resistance of serum.*

Abstract of Protocol.

	Blood.	Serum.	
Diaphragm......................................	1.50 cm.	.88 cm.	—
Cycles per sec................................	2.04·10⁶	—	87,000
R_i' (ohms).....................................	158.7	155.3	
C_r' (m.m.f.)	103	198	
R_r'' (ohms)....................................	157.5	154.2	156.0
C_r'' (m.m.f.)	256	257	
Temperature..................................	21.60	—	
$C_r'' - C_r'$....................................	153	59	
Inductance of coils (10^{-10} henry) $\begin{cases} L_{100}* \\ L_{10} \\ L_1 \\ L_{.1} \end{cases}$	2270 5220 4130 1120	2270 5220 2460 560	
Inductance of leads (10^{-10} henry)...............	−900	—	
Total inductance............................	11,800	9690	
Equivalent capacity (m.m.f.) $\left(\dfrac{\text{total inductance}}{R_r''^2} \, 10^2 \right)$...............	48	40	
Capacity corrected for inductance..............	105	19	
Serum capacity subtracted....................	86		
Correction due to difference in static capacity of diaphragms used for blood and serum........	4		
Capacity of blood (m.m.f)	90		
Resistance of blood (ohms)....................	159		

* L_{100}, L_{10}, L_1, and $L_{.1}$ are the total inductances of the coils used in the hundreds, tens, units, and tenths of units, decades of the resistance box.

A test of the reliability of this method described was obtained by making several series of measurements on cream, where the capacity is zero and the resistance constant.

The following series of experiments will illustrate the procedure and will give a preliminary value for the specific resistance of the interior of a red corpuscle. It is hoped that later publications will show the application of the method to different problems of biological interest and will give also a more extended series of control measurements.

The two experiments here reported were made on the blood of a calf. In the first the original blood was measured and the interior conductivity calculated. In the second the corpuscles of this blood were suspended in an isotonic sugar solution and a new series of measurements was made from which the interior conductivity was again calculated. A control of the method was thus obtained.

These experiments are typical of several others in which different volume concentrations have been used. We have also made a few experiments on the blood of a sheep, for which the specific conductivity of the interior of the corpuscles seems to be about the same as for the corpuscles of a calf—namely 3.5 times the specific resistance of the serum. The variation for different animals of the same species is about ±10 per cent.

The defibrinated blood was obtained from the slaughterhouse. The volume concentrations given in the tables were obtained from the ratio of the resistance of the suspension to that of the suspending liquid using formula (5). In this formula, as we have already stated, there enters the ratio $\frac{a}{b}$ of the thickness of the corpuscle to its diameter.

For this ratio we have used a value of $\frac{1^1}{3}$, a value which in other experiments we found gave volume concentrations in good agreement with those obtained by the hematocrit. In the experiments presented in the tables resistance and capacity were not measured at frequencies under 8700 cycles; in other experiments we have measured at frequencies above 3600 cycles and have found the resistance and capacity to be constant over this whole range of frequencies.

In the tables are given the values of the resistance and capacity which are calculated by equations (1) and (2), selecting the value for R_i which gives the best agreement. There is a deviation between the observed and calculated values which is especially marked in the case of capacity, consisting in a too rapid decrease of the experimental values for the lower frequencies. The character of the deviation suggests the application of equations (3) and (4). As a matter of fact a fairly satisfactory agreement can be obtained by these equations.

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[1] For sheep we used the value $\frac{1}{2}$.

choosing rather small values for $\dfrac{C_0'}{C_0''}$ and rather large for $\dfrac{C_0' \; R_i'}{C_0'' \; R_i''}$,
However, an exact agreement does not seem to be obtained by these equations and since, furthermore, it does not seem possible to attach any definite theoretical meaning to the values of C', C'', R_i', and R_i'' which furnish the best agreement (these values are quite different from what would be expected if C' and C'' corresponded to the two main orientations of a red corpuscle), it is uncertain whether this agreement is of any significance. We have consequently restricted ourselves to the use of formulæ (1) and (2). As stated above it is doubtful if any of the formulæ (1), (2), (3), or (4) is theoretically correct.

The application of (1) and (2) is quite sufficient to obtain a very satisfactory extrapolation of the value of the resistance to infinite frequency. This value is given in the tables as is also the specific resistance of the interior of the red corpuscle as calculated by equation (5) using $\dfrac{a}{b} = \dfrac{1}{3}$.

In the first experiment (Table I) the blood was concentrated to 46 per cent by centrifugation and measurements were made with this suspension. In the second experiment, which was made the next day, 145 cc. of the suspension were diluted with 227 cc. of a 5.4 per cent dextrose solution, the volume concentration of this suspension being consequently $\dfrac{46 \cdot 145}{372} = 17.9$ per cent. The same value of the concentration was also obtained from the resistances of the suspension and of the suspending liquid (using $\dfrac{a}{b} = \dfrac{1}{3}$), showing that the shape of the corpuscles was not changed appreciably by the addition of the dextrose solution. The resistance of the suspension did not change appreciably while the experiment lasted (about 1 hour), being 306.3 ohms at $1.17 \cdot 10^6$ cycles at the beginning of the experiment and 307.8 ohms at the end, values which are identical within the accuracy with which the resistances can be reproduced. We may therefore conclude that no interchange of electrolytes took place between the corpuscles and the dextrose solution during the time of the experiment.[2]

[2] In another similar experiment with sheep blood, when we concentrated the suspension to 72 per cent after the addition of the same dextrose solution to the original blood, we found also that the resistance of the suspension remained constant to within 1 or 2 per cent over an interval of about 1 hour.

The serum of the original blood was diluted by the addition of the dextrose solution to the same extent as was the serum of the blood used in the foregoing procedure.   The resistance of this diluted serum was measured and was found to be equal to the resistance of the suspending liquid which was drawn from the suspension after centrifugation.   This fact proves that no appreciable interchange of electrolytes took place between the corpuscles and the suspending liquid at the moment when the dextrose solution was added.[3]

TABLE II.

Capacity and Resistance of Red Corpuscles of Calf Suspended in a Dextrose Serum Mixture for Frequencies from 87,000 to 4½ Million Cycles.

Jan. 20, 1925.

Cycles per sec. $(\omega/2\pi)$.	Capacity (m.m.f.).	Resistance (ohms).	$\dfrac{73}{1 + (73 \cdot 10^{-12} \cdot \omega \cdot 942)^2}$	$\left[\dfrac{1}{942} \cdot \dfrac{(73 \cdot 10^{-12} \cdot 942)^2}{1 + (73 \cdot 10^{-12} \cdot \omega \cdot 942)^2} + \dfrac{1}{332}\right]^{-1}$
87,000	73	332	73	332
833,000	62	318	64½	319
$1.17 \cdot 10^6$	55	308	58	310
$1.52 \cdot -$	47	299	51	300
$2.04 \cdot -$	37	288	41	288
$3.04 \cdot -$	25	273	26½	271
$3.82 \cdot -$	20	263	19½	264
$4.52 \cdot -$	17	258	15	259
∞	—	(244) extrapolated.	—	245

227 cc. of 5.4 per cent dextrose solution added to 145 cc. of 46.0 per cent suspension from experiment of Table I.

Volume concentration of new suspension: **17.9 per cent.**   Constant of electrolytic cell: **.826.**

Capacity of 1 cc. of suspension for a volume concentration of 100 per cent: $\dfrac{73 \cdot .826}{.275}$

= **220 m.m.f.**

Temperature: **21.8°.**   Specific resistance of suspending liquid: **291.0.**

Specific resistance of inside of corpuscle is **3.5 ± 5 per cent** *times specific resistance of original serum.*

[3] In the case described in Foot-note 2, however, a definite loss of electrolytes took place at the moment when the dextrose solution was added, as was shown by the same test. Correspondingly the resistance of the interior of the corpuscle was in this experiment found to be somewhat lower than for the normal blood.

It will be seen that we obtain the same value for the specific resist-
ance of the interior of the corpuscle in both experiments—namely
3.5 times the specific resistance of the original serum.

The values given in the tables for the capacity of 1 cc. of blood for a
volume concentration of 100 per cent is obtained by the formula
given in the previous paper (formula (1)). The values obtained in the
two cases are the same within the experimental errors, showing that
the capacity is not dependent (or at least is dependent only to a very
slight degree) on the suspending liquid.

<div style="text-align:center">SUMMARY.</div>

1. The variation of the experimental values $(R\ (\omega))$, $(C\ (\omega))$ of
the resistance and capacity of blood for increasing frequencies is
approximately represented by the equation:

$$\frac{1}{R(\omega)} = \frac{1}{R_0} + \frac{1}{R_i} \cdot \frac{C_0^2\ \omega^2\ R_i^2}{1 + C_0^2\ \omega^2\ R_i^2} \tag{1}$$

$$C(\omega) = \frac{C_0}{1 + C_0^1\ \omega^2\ R_i^2} \tag{2}$$

in which R_0 and C_0 are the resistance and capacity of the blood at low
frequency and $\left(\dfrac{1}{R_0} + \dfrac{1}{R_i}\right)^{-1}$ is the resistance of the blood at infinite
frequency. Formulæ (1) and (2) are derived by considering the blood
as equivalent to the system shown in the diagram (a) of Fig. 1.

2. By the application of formula (1) to our experimental data the
value of $R(\infty)$ can be extrapolated with high accuracy. $R(\infty)$
represents the resistance, which would have been obtained at low
frequency, if the membranes around the corpuscles could have been
removed.

3. The specific resistance of the corpuscle interior can be calculated
by equation (5), using experimental values for $R(\infty)$, for the volume
concentration of the blood and for the specific resistance of the serum.

4. The specific resistance of the interior of the red corpuscle of the
calf is found to be 3.5 ± 10 per cent times the specific resistance of
the serum.

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ON SOME GENERAL PROPERTIES OF PROTEINS.

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In this paper we shall consider some general properties of proteins—their denaturation, coagulation, structure, and specificity—on the basis of our knowledge of these properties of hemoglobin (1).

I.

The Coagulation of Proteins.

The coagulation of proteins has been shown to consist of two distinct processes (2, 3). In the first of these the protein is denatured, and in the second the denatured protein is agglutinated or flocculated. Chick and Martin (3) and more recently Lepeschkin (4) have investigated these two processes separately, and it is to them that we owe much of our knowledge in this field. Most of the work has been done on egg albumin. The process of denaturation is greatly accelerated by a rise in temperature, by an increase in hydrogen ion concentration (when the medium is acid), by an increase in hydroxyl ion concentration (when the medium is alkaline), and by alcohol. In general acids increase the rate of denaturation much more than do bases. If the hydrogen ion concentration is kept constant, denaturation proceeds as a monomolecular reaction. The temperature coefficient of the reaction is 1.91 for 1°C. or about 635 for 10°C. The velocity of most chemical reactions is increased two to three times by an increase of temperature of 10°C. The temperature coefficient of denaturation is perhaps the greatest of any reaction that has yet been measured.

Very little is known about the chemical changes occurring in a protein during denaturation. Sörensen does not believe that the

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protein on denaturation gives off any ammonia or other nitrogenous substances (5). "Under these circumstances then, it seems to us most natural to assume that the actual denaturation itself by heating is, like the denaturation with alcohol, not accompanied by any decomposition, but that the decomposition is a secondary process, whereby the egg albumin during and after denaturation is decomposed by the action of hot water." He did, however, show that in denaturation by heating and by alcohol the protein gives off water. So far practically no explanation has been given of denaturation. It is, however, obvious that the protein is radically changed. Whereas it was formerly a hydrophilic colloid it now has many of the properties associated with hydrophobic colloids. Denatured proteins, though very soluble in acids and bases, are readily flocculated at the isoelectric point. The addition of salt extends the limits for complete flocculation.

II.

The Coagulation of Hemoglobin.

The coagulation of hemoglobin is in practically all respects like that of any other protein. Chick and Martin found that dry crystals of egg albumin remained unchanged after 5 hours heating at 120°C. and that dry crystals of methemoglobin were unchanged by subjection for 4 hours to a temperature of 110°C. Hartridge[1] (6) found that the denaturation of *methemoglobin*, like that of egg albumin, is a monomolecular reaction and that the temperature coefficient has the same enormously high value, 1.93 for 1°C. Hemoglobin is first denatured and then coagulated by alcohol. Both acids and bases may be said to denature hemoglobin, for when the solution is later brought to what appears to be the isoelectric point flocculation occurs. The denaturation of oxyhemoglobin has been investigated by Chick and Martin and by Hartridge and that of carbon

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[1] The reader of Hartridge's paper will find that when Hartridge tried to measure the rate of coagulation of "nitric oxide hæmoglobin" he was unable to do so because this compound changed into methemoglobin when warmed. This puzzling result is explained in a recent paper on the combination of nitric oxide with hemoglobin (7).

monoxide hemoglobin by Hartridge. It was found in all cases that denaturation is a monomolecular process, but the temperature coefficients of the reactions were found to be much less than the values found for egg albumin and methemoglobin. The rate of denaturation of HbO_2 was increased 1.3 times for a rise of $1°$ and that of HbCO 1.18 times. These values are much closer to those found for ordinary chemical reactions. It might seem that the process of denaturation of HbO_2 and HbCO was different from that of egg albumin and methemoglobin. But if these experiments are repeated and the changes carefully observed, it appears that Chick and Martin and Hartridge were in reality not measuring the rate of denaturation of HbO_2 or HbCO but rather the rate of conversion of HbO_2 or HbCO into methemoglobin. Spectroscopic examination of the solutions showed us unmistakably that when HbO_2 or HbCO is heated methemoglobin slowly appears and this then coagulates. The "denaturation" of HbO_2 or HbCO consists then of two consecutive reactions, and if the temperature coefficient of the whole process is measured, the result will clearly be the temperature coefficient of the slower reaction. The monomolecular reaction, the temperature of which was measured, was, then, the change from HbO_2 or HbCO to methemoglobin. A detailed analysis of the results of Chick and Martin and of Hartridge confirms this view. Hartridge found that for a given velocity of denaturation he had to heat the HbCO to a higher temperature than the HbO_2. This is to be expected since it is more difficult to convert HbCO than HbO_2 into methemoglobin. Moreover, the temperature coefficient of "denaturation" of HbO_2 was over 1.3 while that of HbCO was only 1.18. It is probable that the true temperature coefficient of the conversion of HbO_2 into methemoglobin is lower than 1.3. It would be expected that a value as high as 1.3 would be obtained because in the test-tube there would actually be a mixture of HbO_2 and methemoglobin so that there would result a value intermediate between the temperature coefficients of the reaction from HbO_2 to methemoglobin and of the denaturation of the latter. In the case of HbCO such a mixture would not so easily occur, and therefore 1.18 is probably very close to the true value for the temperature coefficient of the change from HbCO to

methemoglobin.[2]   In this way we can easily explain why Chick and Martin and Hartridge found that the temperature coefficients of "denaturation" of HbO_2 and HbCO were so much lower than that of egg albumin.   In studying the denaturation of hemoglobin it is therefore on methemoglobin that we should fix our attention, and when we do so we find that the process of denaturation of methemoglobin seems to be the same as that of egg albumin.

III.

The Products of Hemoglobin Coagulation.

When egg albumin is coagulated, denatured egg albumin is first formed.   What substances correspond to denatured egg albumin when hemoglobin is coagulated?   Hoppe-Seyler (8) found that when reduced hemoglobin is coagulated hemochromogen is formed, and that when HbCO is heated in the absence of oxygen CO hemochromogen is formed.   We found that when HbO_2, HbCO, or methemoglobin is coagulated in the presence of oxygen a brown coagulum is obtained.   If some sodium hydrosulfite is put on this and the coagulum is then examined with the microspectroscope it appears that the coagulum was hematin since the reduced product is hemochromogen.   If the alcohol coagulum is washed with water and reduced with sodium hydrosulfite it is found that here too the coagulum consists of hemochromogen.   Indeed Hoppe-Seyler (8) noticed that the red color of muscles preserved in alcohol in museum jars is due to hemochromogen.   When hemoglobin then is coagulated the body corresponding to denatured egg albumin is either hemochromogen or hematin.

IV.

The Nature of Hemochromogen and Hematin.

It has recently been shown (1) that hemochromogen is not merely the iron-pyrrol complex of hemoglobin, but that it is a conjugated

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[2] We have recently (1) presented a theory of methemoglobin formation.   It was supposed that methemoglobin was formed by the following process.

$HbO_2 \rightleftharpoons Hb \rightleftharpoons$ hemochromogen $\longrightarrow$ hematin $\rightleftharpoons$ methemoglobin.
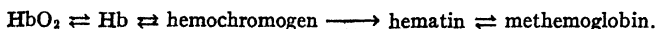
We still think that this mechanism is possible, but the considerations set forth above show that hemoglobin can be more directly converted into methemoglobin.

protein consisting of hem (the iron-pyrrol complex) and globin. Hemochromogen probably has a molecular weight of about 17,000[3] (1). Hemoglobin, whose molecular weight is 67,000 (9) is formed by the polymerization of four molecules of hemochromogen. If this is true, then the process of formation of hemochromogen from hemoglobin or of hematin from methemoglobin is simply a process by which a large protein molecule is depolymerized into four smaller ones. It has been shown above that this process is completely analogous to the process of denaturation of egg albumin, that it is, indeed, the process of denaturation of hemoglobin. On the other hand, hemochromogen has all the properties of a denatured protein. It plays the same rôle in the coagulation of hemoglobin that denatured egg albumin does in the denaturation of egg albumin. Both denatured egg albumin and hemochromogen are formed by heat, alcohol, acids, and alkalies. In both cases acids are more effective than alkalies. Like denatured egg albumin hematin is very soluble in acids and alkalies, but flocculated at the isoelectric point. Just as a solution of denatured egg albumin is much more viscous than the solution of native egg albumin from which it is prepared, so a solution of hemochromogen is much more viscous than the solution of hemoglobin from which it is prepared. A concentrated solution of denatured egg albumin sets to a gel; similarly if alkali is added to a fairly concentrated solution of reduced hemoglobin the hemochromogen formed sets to a gel. Hemochromogen can thus be regarded as denatured hemoglobin. Since the processes of denaturation of egg albumin and hemoglobin are so similar, it is highly probable that exactly the same thing happens in both cases. It would seem, therefore, that when egg albumin is denatured it is depolymerized. Just as hemoglobin can be regarded as a polymer of its denatured form (hemochromogen), so native egg albumin is probably a polymer of denatured egg albumin. In any case, entirely independent of any molecular weight measurements, of any polymerization theory is the general argument that whatever the essential difference between hemoglobin and hemochromogen there is that same difference between the native and the denatured forms of egg albumin.

[3] This value rests on a *preliminary* osmotic pressure determination on acid hematin which Adair made at our suggestion. The exact value may prove to be different, but it is probably not very different.

V.

The Nature of Proteins.

Since the coagulable proteins behave in these respects like egg albumin and hemoglobin the same views can be extended to them.   Thus, among others, we can regard the albumins and globulins as polymerized proteins.   Plant proteins such as edestin (10) form no exception.   Even such a peculiar protein as the Bence-Jones protein is of this nature (11).   The problem that lies before us is to determine the molecular weights of the various denatured proteins and in this way find out for each protein how many denatured molecules polymerize to form a native protein.   We are now doing this by osmotic pressure measurements.

VI.

The Globins and Histones.

The group of proteins known as the histones seems to form an exception to this view of the proteins.   But the exception is more apparent than real.   These proteins are not coagulated by heat (12). They are soluble in acids and rather strong bases.   They are flocculated at their isoelectric points and the flocculation is greatly increased by small concentrations of salts.   It would seem that these proteins have the properties of denatured proteins.   As a matter of fact they probably are denatured, and there is nothing surprising about this, for 10 per cent H_2SO_4 is used in preparing them (13) and such treatment would denature any protein.   Their properties are similar to those of the globins (14), and the latter too must be regarded as denatured proteins.   Here again this is what would be expected, for in the preparation of globin from hemoglobin hematin is first formed, and hematin is a denatured protein.   The globins and histones should not form a separate group of proteins.   They are merely denatured proteins containing large amounts of the more alkaline amino acids

VII.

The Equilibrium between Genuine and Denatured Protein.

It has been shown (1) that in a hemoglobin system there is an equilibrium of this nature:

Hemoglobin $\rightleftharpoons$ hemochromogen.
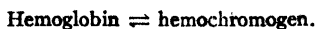
When the system is heated, or when acid, alkali, or alcohol is added to it the equilibrium is shifted to the right; the hemoglobin is denatured. It is only at a moderately low temperature and near the neutral point that much hemoglobin exists. If an alkaline solution of hemochromogen is neutralized, most of the hemochromogen is precipitated. A small amount, however, goes back to form hemoglobin, which can readily be detected spectroscopically. This experiment shows that the denaturation of hemoglobin is a reversible process. The reversibility is, however, masked by the flocculation of denatured hemoglobin—hemochromogen. The denaturation of proteins is regarded as an irreversible process, but it is the flocculation of denatured protein that obscures the reversibility of the process.

The ease with which the equilibrium is shifted varies from protein to protein. Reduced hemoglobin is much more easily denatured, it is known, than is oxyhemoglobin, edestin and euglobulin more easily than egg albumin (15); and in biological reactions other substances may influence the ease of the shift. In the agglutination of bacteria Bordet found that after an agglutinin reacts with the bacteria they can be flocculated only if a small concentration of salt is present. This process (as has often been noted) is in many ways similar to the coagulation of proteins. We merely want to point out that an agglutinin seems to be concerned with the equilibrium between genuine and denatured protein. The equilibrium between genuine and denatured protein will be affected if the denatured protein decreases the surface tension of the solvent. This is the case in the egg albumin system where the denatured protein which is but slightly soluble goes to the surface and is precipitated so that a film of denatured egg albumin appears at the interface (water-gas, or water-collodion). If the film is continually removed by shaking the equilibrium between genuine and denatured egg albumin will be shifted entirely in the direction of the latter, so that practically no more egg albumin remains in solution (16).

VIII.

It would be very interesting to know what group is uncovered when a protein is denatured. The presence of this group on the surface of the molecule converts the protein into an entirely dif-

ferent substance. It would appear to be a non-polar group, for when it is uncovered by denaturation the resulting molecules have much less affinity for water than before; they become less soluble, concentrate at interfaces, and lose water (5). The uncovering of this group also seems to prevent the molecules from manifesting their specificity. The differences between genuine and native proteins as regards solubility, reaction with salts, etc., have often been pointed out. The difference in specificity which, however, is very important from the biological point of view, has scarcely been noticed.

IX.

The Specificity of Proteins.

Perhaps the two most delicate manifestations of protein specificity are the absorption spectra of the hemoglobins and the precipitin reactions of proteins in general. It has recently (17) been shown that the positions of the absorption bands of oxy- and carboxy-hemoglobin vary from species to species and even within the individuals of the same species. These differences have been correlated with the affinities of the hemoglobins for oxygen and carbon monoxide. If these hemoglobins are denatured it is found (1) that the resulting hemochromogens have their absorption bands in the same position—to 1 Å. u.—and that they have the same affinities for carbon monoxide. Denaturing hemoglobin—*i.e.* converting it into hemochromogen—renders it non-specific. The chemical basis of specificity has, however, not been destroyed, for when the denaturation is reversed by converting the hemochromogen into hemoglobin the latter is as highly specific as it was originally. If, for instance, we compare the hemoglobins of rabbit and man, we find that their HbCO absorption bands are in different positions and that their affinities for carbon monoxide are different. The hemochromogens made by treating these hemoglobins with alkali are globin compounds containing *different* globins, and yet their absorption bands are in the same position (within the experimental error of 1 Å. u.) and they have the same affinities for carbon monoxide. When the solutions of these hemochromogens are neutralized the hemoglobins reappear (*i.e.* the denaturation is reversed), and these are found to be exactly as they

had been before. Here, then, we have proteins that are highly specific when native, but not detectably specific when denatured. The same phenomenon has been noticed with the precipitin reaction (18). Whereas genuine egg albumins of different species give rise to highly specific precipitins, the precipitins produced by the injection of denatured egg albumin have only a group specificity. Apparently, then, a protein must be in its native form if it is to be able to manifest its specificity.

If, as was stated above, the globins are really denatured proteins, they should not have any species specificity. Browning and Wilson (19) found that in the complement fixation reaction globins do not show species specificity.

The difference in specificity between genuine and denatured proteins brings us to the highly interesting work of Obermeyer and Pick (20) and Landsteiner (21). They showed that the introduction of chemical groups into proteins endowed those proteins with antigenic properties characteristic of the groups introduced. The species specificity of the protein was lost; the specificity of the group introduced was the only specificity detectable. Landsteiner found "dass man die Azoproteine in gleicher Weise durch Serumreaktionen scharf unterscheiden kann, wie artverschiedene natürliche Eiweisskörper, und sie beweisen, dass die serologische Spezifizität der untersuchten Substanzen wirklich von der chemischen Beschaffenheit der bei der Kupplung eingetretenen Gruppen abhängig ist." Now it is interesting to note that in preparing his protein solutions Landsteiner treated them with an equal volume of 1 N NaOH. Since much less alkali would have sufficed to denature the proteins, there can be little doubt that Landsteiner's proteins were denatured. Since denatured proteins do not show species specificity,[4] it would be expected that if Landsteiner's proteins manifested any specificity at all, within any one group of proteins, *viz.*, the albumins, it would be the specificity of the groups introduced. It was probably the denaturation rather than the introduction of new groups that obliterated the species specificity of the native proteins. There is

[4] Just how much specificity a denatured protein possesses is not known. We are discussing species specificity and not the specificity of groups of proteins.

no evidence that the introduction of a group into a *native* protein would wipe out the species specificity of that protein. Hemoglobin is just such a protein if we consider hem to be the group. Hemoglobin, however, has marked species specificity as shown by direct chemical methods and by serological ones (22).

X.

CONCLUSIONS.

1. The processes of denaturation and coagulation of hemoglobin are like those of other proteins.

2. When hemoglobin is denatured it is probably depolymerized into hemochromogen.

3. When other proteins are denatured they, too, are probably depolymerized. Conversely, native proteins can be regarded as aggregates of denatured proteins.

4. The globins and histones are to be regarded as denatured proteins rather than as a distinct group of proteins.

5. The factors affecting the equilibrium between native and denatured proteins have been considered.

6. A non-polar group is uncovered when a protein is denatured.

7. It has been shown that judged by the two most sensitive tests for the specificity of proteins, it is only when proteins are in the native form that they are highly specific.

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A MICRO DISSECTION OF THE PACHYTENE THREADS OF TRADESCANTIA VIRGINICA L. WITH OBSERVATIONS ON SOME ASPECTS OF MITOSIS.

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PLATES 1 AND 2.

As a report of progress on some work in support of the note on *Tradescantia virginica* L. published in *Science* (1922) under the title Perigenesis, it seems desirable to issue an abstract of some further studies with Chambers' modification of the Barber pipette. Owing to the pressure of other problems it has been impossible to prepare a detailed account of the large amount of material that has accumulated since it involves many drawings and photographs.

Pollen mother cells of *Tradescantia virginica* L. were teased from the anther sacks and suspended in sugar solution or plant sap as described in my paper published with Professor Chambers (Chambers and Sands, 1922–23). A particular study was made of the pachytene threads as illustrated in Plate 2, Figs. 13, 14, 15. These are evidently referred to by Gates (1924) as having been observed recently in *Œnothera* and no doubt form the basis of his comments. It is to be noted that at this stage the fusion of the paired threads, as assumed by those who adhere to the doctrine of parasynapsis, has supposedly been completed. From the knot in Plate 1, Fig. 1, and Plate 2, Fig. 14, the filament loosens and becomes distributed into the nuclear cavity (Plate 2, Fig. 17), there to undergo segmentation in what has been termed second contraction.[1] (Compare Mottier, 1907, Plate 28, Fig. 38, and Davis, 1911, Plate 71, Fig. 14, with

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[1] The use of the term synapsis is avoided. Synizesis might be employed more properly. I should prefer rather to limit synapsis to the phenomena involving the linkage of the chromosomes in a continuous chain in distinction to conjugation of abstricted elements end-to-end so as to cause a reduction of the segments.

Plate 1, Fig. 4 of this paper.)   In *Tradescantia* the rods and rings arise directly from this stage.   They follow segmentation directly without the appearance of any clear-cut diakinesis stage prior to the inception of the equatorial plate phenomena.

With the dissecting needles it is often possible to unravel this heavy skein (Plate 2, Figs. 14, 15, 16), just prior to the stage shown in Plate 2, Fig. 5.   The knot is dissected from the nuclear cavity by cutting away the cellulose membrane together with part of the surrounding cytoplasm so that the skein lies free in the suspension fluid.   The partial operation is shown in the photomicrograph, Plate 2, Fig. 13.   When the knot is normally about ready to loosen and proceed further with segmentation the filament may often be resolved into a continuous thread with or without constrictions. (See also Davis, 1911, Plate 71, Fig. 14.)

The advent of the constrictions seems to vary in individual cells because some seem to have reached the stage of partial segmentation when still quite closely contracted.   Other cells seem to be quite late in reaching this stage and these are the most favorable for manipulation with the needles.

The stages of the formation of the achromatic figure and the events within the filament itself do not always seem to be absolutely synchronized.   Professor Wilson (1925, p. 121) notes that, "We may conveniently treat the history of the chromatic and the achromatic figures as if they were separate, though closely parallel processes."

The constrictions mark the boundaries of the chromosomal elements and, since they do not all appear simultaneously, quite variable lengths of the thread may be cut off.

Ultimately the filament is mapped out into somewhat regular sections.   This may often occur while it is still somewhat contracted. An inspection of Plate 2, Fig. 13, shows the sausage-like forms these units may assume.   Mottier (1907) thought that the rods respliced after segmentation, but the abstrictions observed were, on the contrary, unquestionably incomplete, thus leading to linkages which continue up to and often including metakinesis. (See Plate 2, Fig. 18.)   As the knot loosens and distributes itself into the nuclear cavity, certain of the constrictions become complete abstrictions, thus cutting off segments of the filament united in pairs.   Many

investigators have referred to these elements as bivalents. In *Tradescantia* Belling (*Genetics*, 1925) recently refers to them as such. Except in the case of supernumerary chromosomes, the writer designates them as tetrads, the reason for which will soon appear, although the tetrad nature may not be evident in their morphology. *Many counts here show that the number of so called pairs correspond to one-half the number of somatic chromosomes* (compare vom Rath, 1892).

If these are theoretically tetrads, a sufficient number of univalents will be present to give each of the four resulting pollen grains its haploid quota and the cell itself would therefore be tetraploid. In some forms tetrads, directly recognizable as such, are found as noted below.

For the purpose of greater clearness, the abstriction which cuts off tetrads will be defined as the *primary abstriction*. It results from a completion of the *primary constriction*. By primary constriction, it is not intended to indicate that this is the first which may appear in the continuous thread. The earlier stages have not yet been followed out by dissection. It is the constriction which, by its completion, leads to the primary abstriction.

Beginning with such a stage as Plate 2, Fig. 14 (fixed material), of Plate 1, Fig. 1 (living material), dissections show the stage given in Plate 1, Fig. 2, may sometimes be resolved into a result such as is shown in Plate 1, Fig. 4, in which the filament is continuous and made up of a medulla with an outer rind,—more clearly seen by *intra vitam* staining, as described by me in 1922 and 1923, and also by Chambers (1914, 1925). The structure here is that of the chromosomes figured in my paper of 1923.

Plate 1, Fig. 5, shows a condition found in many cells where the constrictions seem to be either further advanced or the segments, at some points, are less strongly united. In Plate 1, Fig. 6, primary abstriction has occurred at the points marked (*a*). A *secondary constriction* appears at the point (*b*) producing the familiar bivalent figure of Mottier (1903), and many others. In the case of *Fossombronia* (Farmer, 1895), *Pteridophytes* (Calkins, 1897), *Arisæma* (Atkinson, 1899), *Chiloscyphus* (Florin, 1918), and others, another constriction prior to the first metakinesis appears at (*c*) so that, at this time, a picture of morphologically perfect tetrads is presented.

In *Tradescantia*, constriction (*c*) does not normally begin to appear until about the time of the equatorial plate. Sometimes it may be slightly before or after this. By stretching the so called bivalents with the dissecting apparatus, as shown in the photomicrograph (Fig. 9, of my paper with Professor Chambers—Chambers and Sands, 1922–23), the organization within the chromosomal mass at (*c*) becomes somewhat evident at an earlier stage than normal. Plate 1, Fig. 11, of this paper, is a drawing of that figure. The masses of the univalents in both members are partially outlined *m, n, o, p*. When the masses are released from the needle points they resume the bivalent form shown in Plate 1, Fig. 12.

Where closed rings have not been formed, separation on the metaphase spindle of the first division occurs at (*b*) Plate 1, Fig. 6. The constriction here will be referred to as the *secondary constriction and its completion at metaphase, first division, leads to the secondary abstriction*. The constriction at (*c*) will be referred to as the *tertiary constriction*. During the first division, this constriction does not go as far as abstriction, but arrives at this stage in the metaphase of the second division, where univalents, *m, n*, Plate 1, Fig. 11 etc., are separated and a return to the haploid condition is reached (meiosis).

The interkinetic nucleus is therefore diploid (*amphikaryon*). If the mother cell nucleus is diploid, quantitative reduction (assuming no longitudinal splits, etc.) would occur on the first division spindle. However, according to my conception, it has become tetraploid by reason of perigenesis (1922); so that the reason for two reduction divisions becomes apparent. Normally, *i.e.* where no supernumeraries or odd chromosomes due to hybridity are present, there are as many tetrads as haploid chromosomes (vom Rath, 1892, in copepods, calls them segments). It is assumed that perigenesis is suppressed during interkinesis. Some investigators, notably Wilson (1912, *Oncopeltus*), find no return to the resting condition in interkinesis. (See also Richards (1917).)

The inception of the tertiary constriction in *Tradescantia* starts during the equatorial plate stage of the first division and progresses through the anaphase. In the late anaphases or early telophases there may still persist a fine thread of achromatic substance connecting the two masses of chromatic material. Each of these masses is assumed to be univalent and has been so considered by most

other authors. One may find the dyads clearly defined in fixed material, but more often, especially when the chromosomes are short, they are fused so closely by the rigors of fixation that they give pictures like Text-fig. 1 (see also Allen, 1905, Plate 8, Fig. 79).

A constriction analogous to the tertiary constriction has not always been observed in the anaphases and telophases of the first division of the pollen grain nucleus which separates the vegetative and the generative nuclei, although in both cases the chromosomes advance to the poles in the form of V's. The conditions shown in Plate 1, Fig. 10, can easily be observed in either the living condition, *i.e.* in plant juice suspensions, or after *intra vitam* staining by weak methylene blue. Aceto-carmine, as outlined by me in 1923, gives good results. In *Tradescantia* the masses *o* and *p*, also *m* and *n*, Plate 1, Fig. 10, appear about equal.

The free ends *a, a*, Plate 1, Fig. 6, may remain far apart to form rods, Plate 1, Fig. 9. With *intra vitam* staining a clear space is usually observed at (*b*), the secondary constriction. *The same free ends may meet and give true rings* (Plate 1, Fig. 7). (Compare vom Rath, 1892, Rückert, 1892.) Under favorable manipulation with the needles at this time the rings may often be impaled through the central opening. The structure of the elements, in cross-section, is still that of an achromatic cylinder, the same as for the continuous filament of Plate 1, Fig. 4.

Where the primary constriction has not been completed, as at a_x Plate 1, Fig. 8, two tetrads are often left united. This results in an octad linkage. If the free ends *a, a*, of this figure unite, a larger ring than normal is formed—an octad ring. In this fashion, sextad rings may also occur, especially where odd chromosomes are present.

Text-Fig. 1
Fixation fusion of the arms. Anaphase dyads of the heterotypic division in *Tradescantia virginica* L. Flemming's strong solution with triple stain.

The separation of rings such as those in Plate 1, Fig. 8, is accomplished in different ways but mostly as figured by Miyake 1905, Plate 5, Fig. 147, and as I shall describe more fully at another time.

During the telophases of the first heterotypic division, the dyads become completely dispersed simultaneously with the reformation of the nuclear membranes. At its completion, the nuclei are not distinguishable from resting nuclei. The second division shows the usual prophase stages found in vegetative divisions, with some minor differences.

To recapitulate, we have started with a continuous thread that can be demonstrated to be such by the technique described above. In my note entitled Perigenesis (1922) it was pointed out that this thread was a chromatically hollow cylinder, in which the chromatin exists only near the periphery and with the structure of the chromosomes as later (1923) described by me. According to my findings, this chromatically hollow structure of the filament can be recognized in the earliest prophase stages even before parasynapsis is supposed to have occurred. This could not be done with material in any way more coagulated than that obtained by aceto-carmine fixation. Lately, this same structure has been figured by Chambers (1925).

The apparently double nature of the strands and granules of Plate 2, Figs. 6b, 10, 11a, 11b, of Chambers' work has been offered as evidence for parasynaptic pairing. The argument would be more convincing but for the fact that the strands are not double but are, in cross-section (Fig. 7a), cylinders with a plainly defined medulla. The development of the filament from its earliest observable stage is presented by Professor Chambers in Fig. 7a and 7b. The finished structure quite closely agrees with that described in my paper published in *Science* (1922). I cannot conceive of any simple means by which so complex a form could arise from a side by side pairing.

It is quite true that fixation of this structure by chrom-osmoacetic acid will give pictures in optical sections that can be interpreted as double by ignoring the cross-section appearances of spiremes, chromosomes, and telophases.

I therefore look upon the conclusions of Pfitzner, 1882, Müller, 1912, on *Naias marina*, Martens, 1922, on *Paris*, and others, as based on an artifact which from the earliest investigations has lead to conceptions of single longitudinal splits, double longitudinal splits, precocious telophase splits in preparation for the next division, chro-

monemas, development and fusion of internal chromosome vacuoles, chromatic spirals both double and single, and a telophase quadripartite structure developing not only elements that will be separated on the homeotype spindle but at the same time those that will be separated on the spindle of the pollen grain haploid nucleus in forming the vegetative and generative nuclei.

The pairing that *does exist is quite distinct from any of these appearances* and will form the subject of a further paper. Throughout the whole literature of mitosis, the fact that the possible quadripartite structure of the filament (Sands, 1923, Plate 29, Fig. 1), could lead to misinterpretation, has, with but few exceptions, been consistently ignored.

In neither *Tradescantia* nor in *Rhœo*, which has been as fully investigated, was any further evidence for side by side pairing found nor was a longitudinal split apparent. Without the added data from micro dissection it is quite clear from other papers, (Stout, 1912–13, Mottier, 1907, Davis, 1911, Suessenguth, 1921, Gates, 1924, as well as many of the earlier workers) that the relation of the chromosome masses within the spireme is a matter of continuous linkage in a chain.

The process by which this linkage is arrived at, I would term *synapsis* in contradistinction to the reassociation of abstricted elements end-to-end as described by Rückert, 1892, and others. It is seen from this, that, at least for *Tradescantia, division and segregation are everywhere processes of abstriction with subsequent mechanical distribution of the elements.* Where linkage occurs, it is owing to the fact that constrictions may lag, or that abstriction may be suppressed in some cases perhaps permanently.

The data here presented will later be more fully discussed and illustrated. It is obvious that the contentions of Haecker, 1895, Belajeff, 1898, Strasburger, 1900, and others concerning the division and separation of the *idants* cannot apply for *Tradescantia*. *Tradescantia* does not show a longitudinal split in the prophases and hence the use of the term *equational division* should be avoided, except as it implies the separation of sister chromosomes that have arisen by abstrictions in pairs (two univalents) from a continuous filament.

SUMMARY.

A micro dissection of the pachytene threads of *Tradescantia virginica* L. shows that the relation of the chromosomes is a matter of continuous linkage in a chain and that, undoubtedly, division and segregation are everywhere processes of abstriction with subsequent mechanical distribution of the elements.

My thanks are due to Professor R. A. Harper for many helpful suggestions and for reading the manuscript.

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EXPLANATION OF PLATES.

PLATE 1.

Free-hand drawings from the living material of *Tradescantia virginica* L. suspended in *Presssaft* from the plant or in cane-sugar solution.

FIG. 1. Pachytene knot prior to dissection.

FIG. 2. The same as Fig. 1 after cutting away part of the cellulose membrane.

FIG. 3. The knotted thread partially unravelled.

FIG. 4. A continuous spireme resolved from such a stage as Fig. 3.

FIG. 5. Constrictions too far advanced to give such a stage as Fig. 4.

FIG. 6. A bivalent tetrad.

FIG. 7. A tetrad ring.

FIG. 8. An octad ring.

FIG. 9. A tetrad rod.

FIG. 10. Anaphase of the first reduction division showing univalent bodies *m, n, o, p.*

FIG. 11. A drawing of Plate 1, Fig. 9, *J. Gen. Physiol.*, 1922–23, v, 815.

FIG. 12. A drawing of a tetrad such as Fig. 9 of this plate after stretching as in Fig. 11 and releasing again.

Drawings about × 2500.

PLATE 2.

Photomicrographs taken with a Zeiss apochromatic 2 mm. N.A.1.4 oil immersion, compensating ocular No. 8. Bellows at 50 cm. Leitz Lilliput arc, color screens, and panchromatic emulsions. Enlargements × 1600.

The dissection of Fig. 13 was in *Tradescantia Presssaft*. Fig. 14 was an acetocarmine preparation. Figs. 15, 16, 17, and 18 were from material killed in Flemming's strong solution, etc., etc., triple stained.

FIG. 13. The removal of the pachytene knot from the nuclear cavity.

FIG. 14. The same stage as Plate 1, Fig. 1.

FIG. 15. The opening up of the pachytene knot.

FIG. 17. A later stage showing constrictions, in some instances abstrictions and the beginnings of ring formation.

FIG. 18. Incomplete abstriction on the equatorial plate. (Very common in *Rhœo*, an allied genus.)

(Sands: Pachytene threads of *Tradescantia virginica*.)
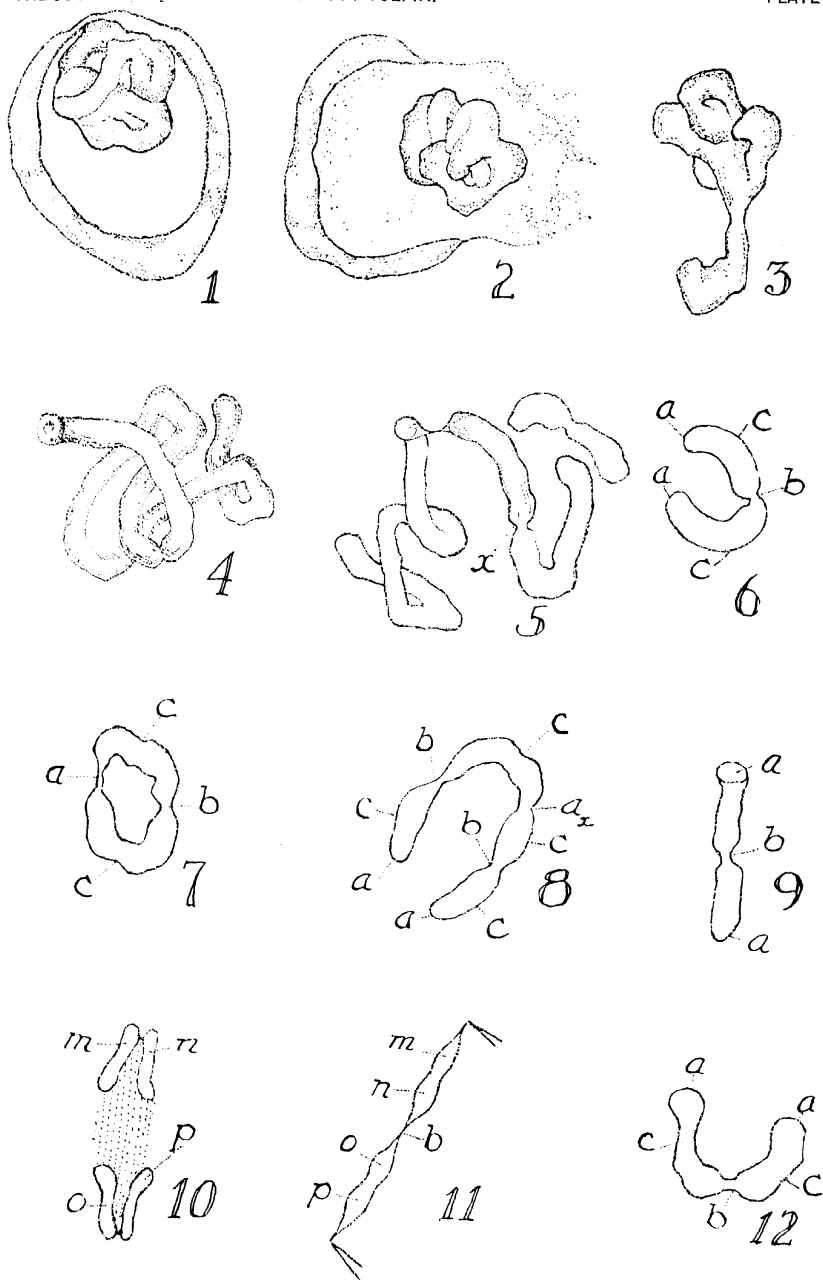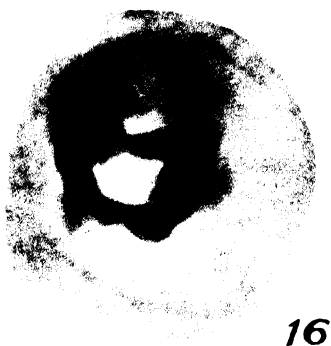
(Sands: Pachytene threads of *Tradescantia virginica*.)

THE CARBON DIOXIDE EXCRETED IN ONE MINUTE BY ONE CENTIMETER OF NERVE-FIBER.

By G. H. PARKER.

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In an earlier paper (Parker, 1925 a) it was shown that the quiescent lateral-line nerve of the dogfish, Mustelus canis (Mitchill), discharges CO_2 at the average rate of 0.0095 mg. of this gas per gram of nerve per minute. This nerve is a pure sensory nerve containing nothing but relatively large medullated nerve-fibers associated with the lateral-line organs. A histological study of it shows no evidence of non-medullated fibers so common in most vertebrate nerves. In consequence of this peculiarity and of the large size of its fibers this nerve is a very satisfactory one for fiber counts and, as its CO_2 output can be measured, it is a favorable object for the problem taken up in this paper, namely, the amount of CO_2 excreted by one centimeter of nerve-fiber in one minute.

Two lateral-line nerves, numbered I and II, were tested in this research. Their CO_2 output was determined by the method described in my previous paper (1925 a, p. 643). The glass respiratory chamber that was used was one that required 0.018,752 mg. CO_2 to change its contents from pH 7.78 to pH 7.36. Under the conditions of the tests this chamber had an average leakage of 0.000,095,2 mg. CO_2 per minute. In nerve I the tests averaged in length 14.8 minutes. Hence the leakage over this period was 14.8-times the amount for one minute or 0.001,409 mg. CO_2. Subtracting this from the amount of the total CO_2, 0.018,752 mg., leaves the weight of this gas produced by the living nerve or 0.017,343 mg. This may be called the organic CO_2 for nerve I (Table I). By a similar method of calculation it can be shown that nerve II whose average period of testing was 12.5 minutes and in consequence of which the leakage must have been 0.001,190 mg., must have had an output of organic CO_2 of 0.017,562 mg.

The organic CO_2 discharged by the two nerves is in part the product of their nervous elements and in part that of their non-nervous constituents, connective tissue, blood-vessels, etc. (Parker, 1925 b). To determine the amounts of CO_2 from these two sources, the proportions of the two classes of tissues, nervous and non-nervous, in the given nerves were estimated. In making these estimates relatively large photomicrographs of parts of cross-sections of the nerves were printed on photographic paper of very uniform thickness. These photographs were cut in such a way as to give a typical sample

TABLE I.

Carbon dioxide in milligrams excreted by two lateral-line nerves, I and II, from the dogfish Mustelus canis (Mitchill). The total CO_2 is given from which is subtracted in the case of each nerve the leakage CO_2 and the non-nervous CO_2. The resulting nervous CO_2 is then divided by the average number of minutes for the tests, the lengths of the nerves in centimeters, and finally by the average number of nerve-fibers in each nerve giving as a final result the weight of CO_2 in milligrams excreted by a centimeter of nerve-fiber in a minute.

Total CO_2..............................	0.018,752	
No. of nerve...............................	I	II
Leakage..................................	0.001,409	0.001,190
Organic CO_2.............................	0.017,343	0.017,562
Non-nervous CO_2........................	0.002,879	0.003,126
Nervous CO_2............................	0.014,464	0.014,436
For 1 min.................................	0.000,977	0.001,155
For 1 cm.................................	0.000,081,42	0.000,075,99
For 1 fiber................................	0.000,000,043	0.000,000,041

of the nerve from its center out to its periphery including its sheath. Such pieces of photographic paper were then weighed, after which all the nervous parts were cut out and they and the remainder were weighed separately. In cutting out the parts the axis cylinders and the medullary sheaths were classed as nervous and the rest of the tissue as non-nervous. On comparing these two parts it was found that in nerve I the non-nervous components constituted 40 percent and in nerve II 47 percent of the whole nerve. The specific weight of the nervous and of the non-nervous components are probably very nearly the same; hence a direct comparison in weight based upon

the percents just given must be approximately correct. Nerve I weighed 128 mgs. and of this 40 percent or 51.2 mgs. must have been the weight of the non-nervous component. Nerve II weighed 140 mgs. and of this 47 percent or 65.8 mgs. must have been the corresponding weight in this nerve.

The rate of CO_2 production for connective tissue and other such materials as made up the non-nervous components of the two nerves was determined by testing in the respiratory chamber sheets of connective tissue taken from the wall of the pericardial cavity of the dogfish. Three such sheets showed respiratory rates of 0.0042, 0.0038, and 0.0035 mg. CO_2 per gram of this tissue per minute and averaged 0.0038 mg. Using this average for nerve I with 51.2 mg. of non-nervous tissue respiring through 14.8 minutes the weight of non-nervous CO_2 given out for this period is estimated to be 0.002,879 mg. For nerve II with 65.8 mg. of non-nervous tissue respiring over a period of 12.5 minutes the corresponding weight of CO_2 is by a similar calculation estimated to be 0.003,126 mg. Subtracting these two weights of non-nervous CO_2 from the appropriate amounts of organic CO_2 leaves for nerve I 0.014,464 mg. and for nerve II 0.014,436 mg. CO_2, to represent the excretions of this gas from strictly nervous sources, the nervous CO_2 in Table I.

The average respiratory rate for connective tissue from the dogfish, 0.0038 mg. per gm. per minute, is about four-tenths that found for the same type of tissue from the frog. Hence my preliminary statement of the amount of CO_2 excreted by one centimeter of nerve-fiber in a minute (Parker, 1925 c), which was based on a nerve determination from a dogfish and a connective tissue determination from a frog, is probably less accurate than that contained in the present paper in which both determinations are from the dogfish.

To excrete the recorded weights of nervous CO_2 required an average period of 14.8 minutes on the part of nerve I and of 12.5 minutes on the part of nerve II. Consequently in one minute nerve I must have excreted 1/14.8 of 0.014,464 mg. or 0.000,977 mg. CO_2, and nerve II 1/12.5 of 0.014,436 mg. of 0.001,155 mg. CO_2. These are the weights of nervous CO_2 excreted per minute by each nerve (Table I).

To ascertain how much CO_2 is excreted by a centimeter of nerve

it is necessary to take into account the length of the nerves. Nerve I excreted 0.000,977 mg. CO_2 per minute and was 12 cm. long. Hence one centimeter of it must have excreted on the average 0.000,081,42 mg. CO_2. Nerve II excreted 0.001,155 mg. CO_2 per minute and was 15.2 cm. long. One centimeter of this nerve must, therefore, have excreted 0.000,075,99 mg. CO_2 (Table I).

To ascertain how much CO_2 a single nerve-fiber one centimeter long excretes in one minute, it is necessary to determine the number of nerve-fibers in each nerve. The lateral-line nerve of the dog-fish tapers with great regularity from its anterior to its posterior end as it gives off fibers to the lateral-line organs. It is therefore necessary to make an estimate of the number of fibers by averaging the totals for each end of the nerves. Cross-sections were prepared from each end of each nerve and the fibers were counted in these sections. The nerves had been preserved in alcoholic formol. On treating this material with osmic acid the nerve-fibers, especially the axis cylinders, were brought out with great distinctness and could be counted with ease. For the preparation of this material I am under obligation to Mr. Benjamin Kropp whose excellent technique greatly facilitated my work.

Each nerve in cross-section is seen to be broken up into numerous small bundles and these bundles can be conveniently further subdivided. Thus small well circumscribed areas can be established and marked out under the camera lucida. Each of these areas contained from twenty to thirty fibers in cross-section and could be worked over under the microscope with and without the camera till the count was reasonably certain. In this way sections from the distal and from the proximal ends of each nerve were completely counted. In nerve I the proximal cross-section contained 2115 fibers and the distal 1708 fibers with an average of 1911.5 fibers. In nerve II by a similar method the average was found to be 1841 fibers.

If one centimeter of nerve I excretes 0.000,081,42 mg. CO_2 in one minute and contains an average of 1911.5 nerve-fibers, then one fiber in such a stretch excretes on the average 0.000,000,043 mg. CO_2 per minute. Similarly in nerve II with 1841 fibers the weight of CO_2 excreted per minute per centimeter of nerve-fiber was calculated to

be 0.000,000,041 mg. (Table I). Since these two determinations average 0.000,000,042 mg., it may be concluded that one centimeter of lateral-line nerve-fiber excretes on the average 4.2×10^{-8} mg. CO_2 per minute. It might be surmised that so very small a weight of CO_2 as that just stated would approximate molecular proportions, but it can be shown easily on the basis of Avogadro's number, 6.06×10^{23}, that so minute an amount of CO_2 as 4.2×10^{-8} mg. contains over 578-thousand-million molecules of this gas.

SUMMARY.

One centimeter of nerve-fiber from the lateral-line nerve of the dogfish is estimated to excrete on the average 4.2×10^{-8} mg. CO_2 per minute.

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THE SHAPE OF THE MAMMALIAN ERYTHROCYTE AND ITS RESPIRATORY FUNCTION.*

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In a note on the shape of the mammalian red cell, Hartridge (1) has suggested that the erythrocyte has a form which is a compromise between that of a sphere and that of an infinitely thin disc. Gas diffusing from the surface of either of these figures would reach the central regions at the same time. The sphere, however, offers a small surface compared to its volume, while in the thin disc, the surface layers forming the envelope would be greatly increased at the expense of the contents. In the disc, moreover, gas would gain access too readily at the ends, so that the peripheral regions would be reached soonest. A body of the shape of the normal red cell meets the difficulties of the problem, for not only is the surface comparatively large for the volume, but the ends are thickened, so that gas diffusing from the surface reaches the central regions in a uniform manner.

The red cell being greatly concerned with gas transportation, it is obviously important to consider whether its special shape offers any special advantages. This we propose to do in this short paper, thus following up the suggestion of Hartridge.

Consider two equal and isolated sinks, S_1 and S_2. They will set up lines of flow,

$$\cos \theta - \cos \theta' = \text{Constant},$$

and lines of equal velocity potential

$$\frac{1}{r} + \frac{1}{r'} = \text{Constant}.$$

Supposing that a gas starting from a line of equal velocity potential passes along a line of flow, it will thus pass at right angles to all the lines of equal velocity potential which it traverses, and finally reach one of the sinks.

If the strength of the sink S_1 be m_1, and that of S_2 be m_2, then, if the sinks are apart by a distance a, the lines of equal velocity potential will be

$$\frac{m_1}{r_1} + \frac{m_2}{r_2} = \frac{k}{a}.$$

Such an equation gives, for various values of k, a series of curves known as the equipotential curves of Cayley, who first described them in 1857 (2). In our case, the strength of the sinks is equal, or $m_1 = m_2$.
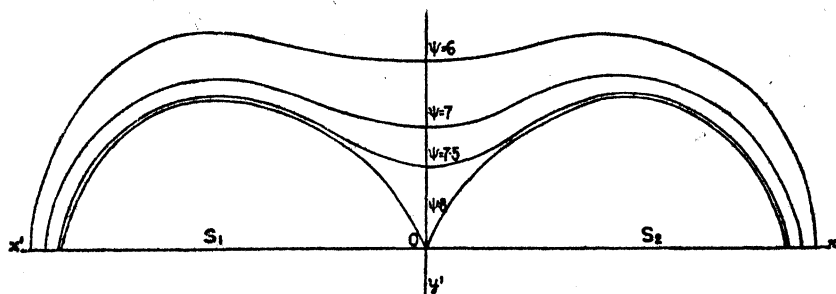
FIG. 1. Equipotential lines for various values of ψ, the velocity potential. Lines above the abscissa alone are shown; the lines below the abscissa are identical with those above.

Taking as a convenient strength that of 24 units, and placing the sinks 12 units $= a$, apart, one can plot these equipotential lines for various values of ψ, the velocity potential. This is done in Fig. 1, lines for a series of values of ψ being shown. The method of plotting these lines is similar to that used for obtaining lines of equal potential due to two similar and equal magnetic poles (3): in the figure, lines above the abscissa alone are shown, the lines below the abscissa being identical with those above. These lines are, of course, identical with the lines of equal velocity potential produced by two equal sources.

It will be seen that as ψ decreases, the line representing equal values of ψ passes from an oval (not shown in the figure) to a curve presenting a concavity; as ψ continues to decrease, the concavity becomes

deeper, and ultimately the curve splits into two loops with a double point on the abscissa mid-way between the sinks. A further decrease in ψ causes the curve to assume the form of two ovals, one lying about each sink, and finally, when the velocity potential is zero, the curve becomes two points, S_1 and S_2. From the equation of the curve, it will be seen that, if the sinks are equal in strength,

    (1) when $k > 4m$, the curve consists of a large oval, with or without biconcavities;
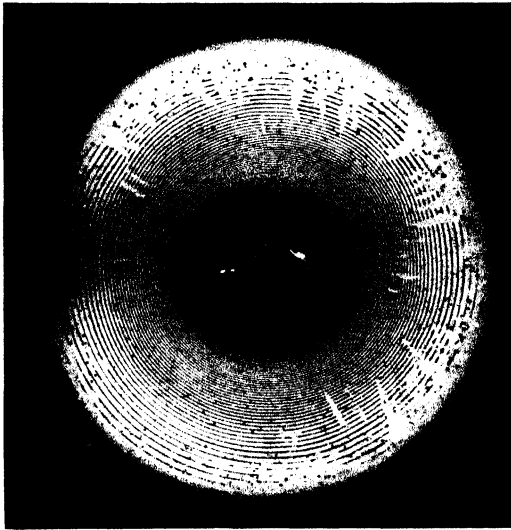
FIG. 2. Lines arranged around one "source."

    (2) when $k = 4m$, the curve has a double point;
    (3) when $k < 4m$, the curve consists of two ovals.

It will further be observed that the series of lines for equal values of ψ may be regarded as a wave surface converging on S_1 and S_2, as ψ decreases, and hence particles of gas commencing to move towards the sinks from any one line of equal velocity potential will arrive at the sinks at the same moment.

Apart from the theoretical aspect of the case, curves very similar to these equipotential curves of Cayley can be produced by a very simple experimental device, in which the conditions are analogous to

those which we are considering. A uniform ground of gelatin containing potassium chromate is formed on a glass plate, and on this is placed either one or two drops of 4 N silver nitrate, the drops being made as small as possible. The whole preparation is then kept in a moist chamber. As the silver salt diffuses from the droplet into the gelatin, a series of rings appears arranged round the drop or drops. These are shown in the photographs, for which I have to thank Dr. W. W. Taylor, who first called my attention to the Liesegang phenomenon. In the first case, a single drop was used; in the second, two drops a small distance apart. The lines which appear etched on the ground are lines of equal salt concentration, analogous, in a general
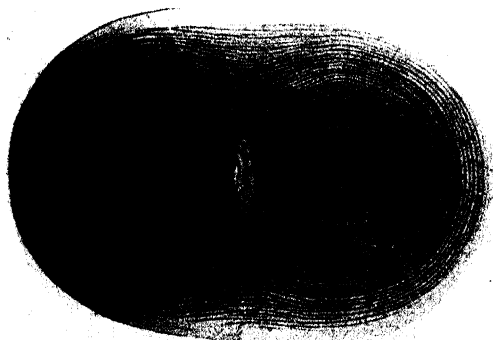
Fig. 3. Lines arranged around two "sources."

way, to lines of equal velocity potential. It will be seen that in the case of the single drop, these lines are circles, but that when there are two drops, which we can look upon as two equal and similar sources, the lines are similar to the equipotential curves mentioned above. Reversing the process, and making the sources become sinks, will plainly give the same form of lines around the sinks as round the two sources. This experiment must, of course, be taken merely as an illustration, for the primary condition that the sources shall be points is not fulfilled; nevertheless, it shows the general result very well.

Selecting the curve $\psi = 7.5$, and rotating it about its minor axis

yy', we obtain a solid of a shape approximately that of the erythrocyte. In the process, the sinks S_1 and S_2 become a circular sink, and the equipotential line which forms the curve becomes the equi-velocity potential surface of the solid of revolution. Gas starting from any point of this surface and moving inwards at right angles to it along lines of flow will reach this circular sink in the same time, irrespective of the point on the surface from which it started. Further, taking account of Fick's law of diffusion across a membrane, it will be seen that any line of equal velocity potential must also be a line of equal gas concentration; since the surface of the solid of revolution is a surface of equal velocity potential, as much gas will pass across any one unit of the surface in a given time as will pass across any other unit. The gas thus passing across the membrane will proceed along lines of flow, and reach the circular sinks.

Now, turning to the erythrocyte, which may be imagined as containing no oxygen, and floating in a fluid containing a certain quantity of dissolved gas, it is obvious that the surface of the cell must be one of equal gas concentration, and that therefore gas will pass across the surface towards the inner parts of the cell to form a series of surfaces of equal gas concentration, and of equal velocity potential. The gas starting from the equipotential surface of the cell must converge on the circular sink, which will be reached simultaneously by all particles of gas which start from the surface at the same moment. If, then, the form of the erythrocyte were that of the solid produced by the rotation of the curve $\psi = 7.5$, or, indeed, any curve of equal velocity potential, about yy', that form would be one peculiarly suited to the even and orderly distribution of gas throughout its contents.

The passage of gas from the surface of a solid of this form results, of course, in a progressive loss of strength of the circular sink. This, however, in no way alters the general nature of the diffusion process, for if, by gas entering the cell, the strength of the sink becomes halved, then the surface $\psi = 7.5$ will become the surface $\psi = \dfrac{7.5}{2}$ but will remain of the same form. Alterations in the numerical strength of the sink, such as would be produced by gas entering the cell, do not, therefore, affect the form of the surface, but only its numerical strength. Nor does the fact that the sink may become a source

influence the manner of diffusion of gas across the surfaces of equal velocity potential, except as regards the direction of the flow.

It now remains to be seen to what extent the equipotential curves of Cayley can provide a satisfactory fit to the shape of the red cell, for the nearer the shape of the cell to that of the curves, the better will it be adapted for the purposes of the flow of gases within it. The goodness of fit of the curves may be tested in two ways: by comparing the dimensions of the curves with those of the cell, as ascertained by measurement, and by comparing the volume enclosed by the different equipotential curves with that of the red cell. It is, to begin with, clear that the only one of the equipotential curves with which we need concern ourselves is $\psi = 7.5$, curves for other values of ψ being obviously inapplicable to the shape of the cell.

Photomicrographs show that the erythrocyte is not quite so rounded at the ends as is this curve, and that its concavity is not quite so deep. It is admittedly difficult to judge of these matters from even the most carefully taken photomicrographs, for one is very apt to be deceived, as regards the general contour of the cell, by the slight flattening which occurs when the cells are in rouleaux—it being difficult to obtain them in profile unless thus arranged—and also to underestimate the depth of the concavity. Nevertheless, one cannot help coming to the conclusion that the equipotential curves are too great at their greatest ordinates, and too narrow at the point where $x = 0$, to represent the red cell with great accuracy.

The crucial test, however, lies in the comparison of the volume enclosed by the rotated curve $\psi = 7.5$, with the surface of the erythrocyte. The equipotential curve lends itself to neither rectification nor quadrature, which is not surprising, as it is of the eighth degree, and very complicated in Cartesians. The volume of the solid of revolution produced by its rotation about yy' must therefore be found by graphical methods and the use of the theorems of Pappus. In a like manner we can find the surface of the solid of revolution.

For the curve $\psi = 7.5$, rotated about yy',

Major axis..	20 units.
Area of quadrant....................................	34.45 units².
C. gr. of area......................................	5.33
Arc of quadrant.....................................	13.0 units.

C. gr. of arc........ ....   5.66
Volume of solid.................................... 2308 units³.
Surface of solid.................................... 925 units².

Reducing this to the scale of a human red cell of diameter 8.8μ, the volume of the solid of revolution works out at approximately $196\mu^3$. Now the volume of the erythrocyte can be obtained with considerable accuracy by converting it into the spherical form by immersion in isotonic saline (4, 5) and calculating its volume from measurements while it is in this spherical state. Such a procedure gives, as an average volume for the human red cell, about $110\mu^3$, and, even if experimental error be allowed for, to the fullest extent, no figure greater than $120\mu^3$. Comparing this with the volume for the solid of revolution derived from the curve $\psi = 7.5$, it is plain that the discrepancy, which amounts to about 60 per cent, is too great to be allowed. It must therefore be concluded that the shape of the erythrocyte cannot be described by one of the equipotential curves of Cayley, convenient though such a description would be: on the other hand, the general resemblance between the erythrocyte and these solids with equipotential surfaces should not be overlooked, for the more complete the resemblance, the better is the cell adapted in its function of gas exchange. The resemblance which does in fact exist is such as to make the efficiency of the cell very great compared with that of most other figures of the same volume, and very much greater than that of a spherical form, for, in order to obtain the same rapidity of gas diffusion for the same volume distributed in spheres, we would require approximately nine spheres each of one-ninth the volume. Even allowing for the fact that there is not a perfect agreement of the form of the red cell with that of a body with an equipotential surface, the efficiency of the cell for gas interchange is very great, as Hartridge has noted.

Taking a body of exactly the form of the red cell, it ought to be possible to determine the manner in which gas will pass throughout its contents by proceeding on lines similar to these. The lines of flow and the surfaces of equal velocity-potential will be nearly, but not quite, the same as those for a body with a surface given by the revolution of one of Cayley's curves, and the diffusion of gas will take place according to the same general scheme. But the sinks will have to be

postulated as something other than points, and in their neighbourhood the phenomena will become very complex: we have therefore not thought it worth while to pursue the study further, as in purpose this paper is more suggestive than complete.

This consideration of the advantage produced by the form of the cell approximating to that of a solid with an equipotential surface does not, of course, offer any explanation as to why this form should be taken up. The rotation of the line $\psi = 8$ about yy' would produce an even more adapted solid, but yet the form of this solid is far from that of the red cell. The reason for the assumption of the special shape of the erythrocyte is to be sought along very different lines, and though the approximation to the form of a solid with an equipotential surface may be very interesting, that approximation may be nothing but a coincidence.

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THE EFFECT OF THE H ION CONCENTRATION ON THE AVAILABILITY OF IRON FOR CHLORELLA SP.*

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The H ion concentration of the culture medium is now recognized as being an important factor in the growth of all classes of plants. In certain special cases this may even become the limiting factor for growth. However, in experiments with higher plants growing in soils of different pH values a correct interpretation of results is difficult, because it is frequently impossible to isolate the direct effects of the hydrogen ion concentration from other contributing factors. The following experiments with a unicellular green alga, *Chlorella sp.*, growing in mineral nutrient solutions under pure culture conditions, were performed in an effort to obtain a more exact analysis of the effect of the H ion concentration on the rate of growth of a green plant.

Methods.

Uniform amounts of a pure culture of *Chlorella* were introduced into liquid culture solutions in pyrex Frlenmeyer flasks of 150 cc. capacity. For each culture 50 cc. of a mineral nutrient solution was supplied. In each experiment the salt content of the several solutions was the same in all cultures, except for the varying amounts of the phosphate buffers used to obtain the range of pH desired. The dry weight of the crop produced was used as the criterion of growth.

Growth of Chlorella in Complete Nutrient Solution at Varying H Ion Concentrations.

Each culture solution in this series of experiments consisted of equal portions of a mineral nutrient solution, designated Solution *A*, and of a phosphate buffer mixture, Solution *B*. Solution *A* contained 2.95 gm. $Ca(NO_3)_2$, 0.4 gm. $MgSO_4$, a trace of $Fe_2(SO_4)_3$, and
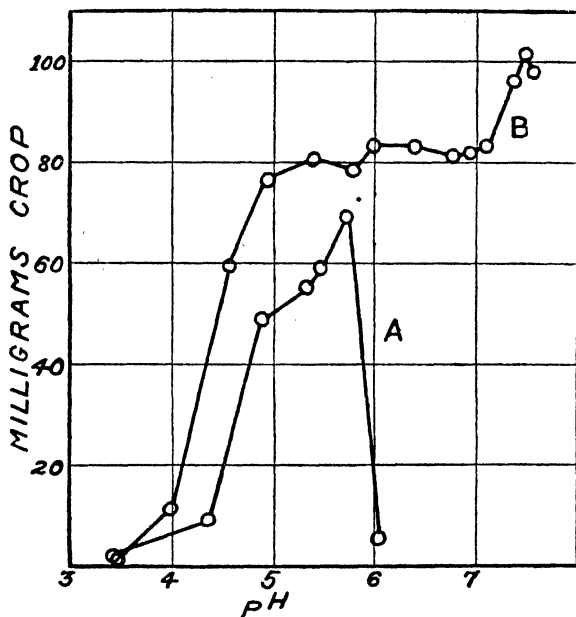
FIG. 1, *A*. Growth-pH curve with normal culture solution from which the iron was precipitated at the higher pH values.

B. Growth-pH curve with culture solution in which the calcium of the normal culture solution was replaced by ammonium and in which the iron was held in solution in the more alkaline cultures by the addition of sodium citrate.

20 gm. glucose per liter. The buffer mixtures (*B*) were prepared from $\text{м}/7.5$ solutions of KH_2PO_4, K_2HPO_4, and H_3PO_4 in such proportions as to give a range of H ion concentrations from pH 2.8 to pH 7.13. Four replications of each pH were prepared; one was used for the initial pH determinations and the remaining three in each case were inoculated. The H ion determinations were made electrometrically using a bubbling type of electrode, and were checked

by Gillespie's colorimetric method. The cultures were inoculated with ½ cc. of a suspension of the algal cells in a balanced solution consisting of 95 cc. of 0.6 per cent NaCl and 5 cc. of 2.2 per cent CaCl₂. The usual aseptic precautions were observed throughout the preparation of solutions and the inoculations.

2 weeks after inoculation determinations were made of the dry weight of the crop of each culture and of the final H ion concentration of each solution. The contents of individual culture flasks were centrifuged in large pyrex test-tubes, a portion of the clear supernatant liquid poured off for pH determination, and the algal cells then thrown on the asbestos mat of a Gooch crucible. The weight of the crop was ascertained after drying *in vacuo* at 80°C. for 18 hours. The results obtained in one of the experiments are presented graphically in Fig. 1, *A*.

The data for the final pH determinations showed that the H ion concentrations of the solutions remained very constant even where there was considerable growth of the alga. The average crop determinations, plotted in Fig. 1, *A*, show so far as this experiment is concerned that maximum growth appeared at pH 5.7. The acid limit may be placed at about pH 3.4 and the alkaline at about pH 6.1. Substantially the same results were obtained in two duplications of this experiment.

Precipitation of Salts in the Nutrient Solutions.

In the above experiments all the solutions on the acid side of pH 5.7 were perfectly clear, whereas considerable precipitate occurred on the alkaline side of this point. The precipitate consisted chiefly of magnesium and calcium phosphates, and it seemed possible that the iron, supplied as ferric sulphate, was also largely thrown down. Lack of growth in solutions more alkaline than pH 5.7 could not be interpreted, therefore, as due to a direct effect of the H ion concentration, but might be rather the result of the unavailability of some essential mineral element. To test this assumption a considerable quantity of the complete nutrient solution was prepared, using a buffer mixture which gave an initial H ion concentration of pH 6.8. The copious precipitate which formed was filtered off and the clear filtrate adjusted to pH 5.5 with concentrated HCl of low iron con-

tent. This adjusted solution, though apparently near the optimum pH, permitted only a slight growth when inoculated with *Chlorella*. The addition of ferric sulphate increased the growth three fold, and of magnesium sulphate two and a half fold, whereas growth was depressed by the addition of calcium chloride. In the unadjusted solution at pH 6.8 no growth of the organism was apparent. These results indicate that in solutions of pH 6.0 and above the precipitation of iron is a factor in the growth of *Chlorella*.

The Solubility of Ferric Iron in Buffered Culture Solutions.

In order to determine the extent of the precipitation of iron quantitative tests were performed on filtered buffer solutions, and at the same time an attempt was made to keep the iron in solution by means of salts of certain organic acids. For this purpose a phosphate buffer mixture having a pH value of 7.0 was prepared and iron was added to this as ferric sulphate. To various portions of this solution sodium citrate, sodium tartrate, and sodium potassium tartrate were added. At the time of mixing all these solutions were turbid except the ones to which sodium citrate had been added. After standing overnight the solutions were filtered and the clear filtrates tested for iron by the procedure of Marriott and Wolf.[1] This method, slightly modified by the writers, was found to be highly satisfactory for the determination of such small amounts as 0.0005 mg. ferric iron. The tests showed that about $\frac{1}{100}$ of the iron was left in the phosphate buffer solution to which no organic salt was added, whereas in the solutions containing sodium citrate all the iron remained in solution. The two tartrate salts were not effective in holding the iron in solution. Additional tests showed that as little as 0.005 gm. of sodium citrate in 50 cc. of buffer solution was sufficient to hold all the iron in solution at pH 7.0, and further that the presence of magnesium sulphate did not cause its precipitation. When, however, an attempt was made to keep the iron in an available form in the complete nutrient solution by the addition of sodium citrate it was found that in all solutions of pH 6.0 and above, this was not effective. The loss of the iron in

[1] Marriott, W. McK., and Wolf, C. G. L., The determination of small quantities of iron, *J. Biol. Chem.*, 1905–06, i, 451.

these more alkaline solutions may be accounted for by the formation of an amorphous precipitate of calcium phosphate on which the iron was apparently adsorbed. It was evident therefore that with a considerable amount of calcium present it would be impossible to hold the iron in a form available for growth in solutions of pH 6.0 and above.

Effect of the H Ion Concentration on the Growth of Chlorella in Solutions Lacking Calcium.

As it has been shown by Schramm[2] that very little if any calcium is necessary for the growth of *Chlorella*, a series of solutions of differing pH was prepared from which calcium was entirely omitted. The same procedure was followed as in the first experiments recorded in this paper. Solution *A* in this case, however, consisted of 1.0 gm. NH_4NO_3, 0.4 gm. $MgSO_4$, 0.4 gm. sodium citrate, 0.8 mg. $FeCl_3$, and 20 gm. glucose per liter. The ferric chloride was added from a standard solution of iron wire in hydrochloric acid. The usual buffer mixtures were designed to give a range of pH from 3.5 to 7.6. The completed solutions were all clear, and with sodium citrate present the iron was neither precipitated chemically nor removed by adsorption. Initial iron tests showed as much soluble ferric iron in the most alkaline solution (pH 7.5) as in the most acid one (pH 3.5). Six replications of each solution were prepared, five of which were inoculated, and the sixth used for the initial pH and iron determinations.

Crop determinations made at the end of 2 weeks showed the usual rapid increase in the amount of growth from pH 3.5 to pH 5.4. Between pH 5.4 and 7.15 the rate of growth was approximately the same in all cultures. This was followed by a second increase in the dry weight of the crops at pH 7.4 and 7.5. The results are shown graphically in Fig. 1, *B*.

The final H ion concentration determinations showed in nearly all cases that there was a slight increase in the acidity of the solutions. Estimations of the residual ammoniacal and nitrate nitrogen

[2] Schramm, J. R., A rapid and delicate method involving pure cultures of algæ for mineral nutrition studies. Paper presented before the Botanical Society of America, Boston Meeting, Dec. 29, 1922.

in the solutions demonstrated that this change in reaction was due to a differential absorption of the ions of ammonium nitrate.

CONCLUSIONS.

The data obtained in these experiments indicate clearly that unless the necessary precautions are taken to keep the iron of the culture medium in solution the results obtained by varying the H ion concentration will not represent the true effect of this factor on growth. The availability of iron in nutrient solutions has been the subject of numerous recent investigations and it is now known that iron is precipitated at the lower hydrogen ion concentrations, that the iron of certain iron salts is less likely to be precipitated than that of others, and that certain salts of organic acids tend to keep the iron in solution. In general, ferric citrate seems to be the most favorable source of iron. In addition to chemical precipitation, however, it is also possible for the iron to be removed by adsorption on an amorphous precipitate such as calcium phosphate. As this precipitate is frequently formed when nutrient solutions are made alkaline, this may account for the discordant results reported in the literature as to the availability of certain forms of iron. By omitting calcium from the culture solution iron can be maintained in a form available for growth in alkaline solutions by the addition of sodium citrate. In such solutions the maximum growth of *Chlorella* occurred at pH 7.5. The alkaline limit for growth has not been established as yet.

In investigating the availability of iron at varying concentrations of the hydrogen ion, changes in the pH value of the solution during the course of an experiment should also be taken into account. This is especially important in unbuffered solutions. The differential absorption of the ions of ammonium salts may cause a marked increase in the hydrogen ion concentration, which in turn will cause an increase in the solubility of iron. In strongly buffered solutions as used in these experiments this effect is slight.

THE EFFECT OF RADIOACTIVE RADIATIONS AND X-RAYS ON ENZYMES.

IV. THE EFFECT OF RADIATIONS FROM RADIUM EMANATION ON SOLUTIONS OF INVERTASE.

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In our studies[1, 2] concerned with the effect of the radiations (beta and gamma) from radium emanation, in equilibrium with its radioactive products, on enzymes in solution we have observed that trypsin and pepsin are inactivated. It has been possible to follow the course of these radiochemical reactions quantitatively; and, under the conditions of experiment employed by us, we have found that the change in the logarithm of the concentration of active enzyme is a linear function of the product of two variables, namely, the average activity or power of the radioactive source, P_a, expressed in terms of the unit curie-power,[3] and the time of exposure, t, expressed in hours. This product has the dimensions of energy and represents a single variable quantity which we designate by W and express in terms of the energy unit curie-power hour; *i.e.*, $P_a t \equiv W$. The relation between the chemical change observed and the variable W is simply expressed by the equation

$$\log Q - \log Q_0 = - kW \tag{1}$$

where Q_0 represents the initial concentration of active enzyme, and Q the concentration of active enzyme after an irradiation for a given increment of energy, W. The concentration terms are expressed in arbitrary units. The logarithms are to the base e.

[1] Hussey, R. G., and Thompson, W. R., *J. Gen. Physiol.*, 1922–23, v, 647.

[2] Hussey, R. G., and Thompson, W. R., *J. Gen. Physiol.*, 1923–24, vi, 1.

[3] Hussey, R. G., and Thompson, W. R., *J. Gen. Physiol.*, 1923–24, vi, 7.

In order to determine whether the mode of behavior described in the case of trypsin and pepsin when irradiated with the radiations mentioned is a fact of wider application we have thought it desirable to extend our observations by similar experiments with another enzyme. The selection of a third system was influenced by a desire to employ some other type of enzyme than a proteolytic one, but one for which precise methods were available for determining its activity. It has been our good fortune to obtain from Professor J. M. Nelson a preparation of invertase for the purpose of our experiments.

Experimental Results.

The invertase preparation employed in our experiments is designated as Normal Invertase 8. For details regarding its preparation and general characteristics the reader is referred to papers by Professor Nelson and his coworkers.[4-7] The concentration of active invertase, Q, was determined from the relation $Qt = f_{(p)}$, where t is the time required for a given percentage inversion of sucrose, p.[8] Preliminary observations were made in which the inversion of two different preparations of sucrose was followed. One of these was obtained from the U. S. Bureau of Standards where it was prepared in accordance with the technique described by Bates and Jackson. The other preparation was Merck's Blue Label sucrose. No significant difference was observed in the form of the inversion curve[9] and consequently since

[4] Nelson, J. M., and Hitchcock, D. I., *J. Am. Chem. Soc.*, 1921, xliii, 2632.

[5] Nelson, J. M., and Vosburgh, W. C., *J. Am. Chem. Soc.*, 1917, xxxix, 790.

[6] Vosburgh, W. C., *J. Am. Chem. Soc.*, 1921, xliii, 219, 1693.

[7] Nelson, J. M., and Born, S., *J. Am. Chem. Soc.*, 1914, xxxvi, 393.

[8] In order that the symbols employed throughout this series of papers be consistent we have employed Q to represent the enzyme concentration instead of n as used by Nelson and Hitchcock.[4] One unit concentration of invertase ($Q = 1$) was arbitrarily taken as the concentration of a solution half the strength of the stock solution. 5 cc. of such a solution in 100 cc. of sucrose reaction mixture gave an average value for n of 1.78×10^{-2}. Accordingly the n value of the original solution (50 per cent of stock) must have been 3.56×10^{-1}, which is equal to one arbitrary Q unit.

[9] It is of interest to note that after the destruction of part of the invertase the form of the sucrose inversion curve was not altered although the rate of inversion was reduced.

Merck's preparation is more readily available it has been used throughout the experiments to be reported in this paper.

In all of our experiments the composition of the reaction mixture was as follows:

Sucrose concentration........... 10 gm. per 100 cc.
Buffer (acetate) concentration.... 0.01 molar
Invertase concentration......... 5.00 cc. of invertase dilution (50 per cent by volume) per 100 cc.

In each experiment a control test was run simultaneously with each test for determining the effect of irradiation on the invertase. The progress of the sucrose inversion was followed in all essential details in the manner fully described by Nelson and his coworkers. For these details the reader is referred to the papers already mentioned by these investigators.

The procedure followed in irradiating the samples of invertase was similar to that described by us for pepsin.[2] The radium emanation was contained in a small glass bulb and the amounts employed varied between 100 and 500 millicuries.

EXPERIMENTAL RESULTS.

Several experiments were made in which 8.12 cc. of a 50 per cent dilution of the stock invertase were irradiated in a spherical glass bulb as previously described for the irradiation of pepsin in solution, the value of W being varied. The results obtained from such an experiment are shown in Table I, where it will be observed that the value of the mean reaction speed coefficient is sensibly constant, as is required to satisfy the conditions stated in equation (1). Thus it is evident that the principles established for the radiochemical inactivation of trypsin and pepsin apply equally well to invertase.

So far in our experiments with trypsin and pepsin[1, 2] we have reported only the results obtained where the volume of enzyme solution irradiated was constant. In this paper we present in addition the results of experiments wherein this volume was varied. If, as we have assumed to be the case in our previous experiments,[1] the layer of fluid around the emanation bulb is of sufficient thickness to absorb practically all of the beta radiation, and the speed of diffusion is great

enough to maintain uniform concentration of enzyme throughout the liquid system, and in addition that the effect of the gamma radiation is negligible; it would be expected that the rate of change in the logarithm of Q with respect to W would vary inversely with the volume, *i.e.*

$$-\frac{d \log Q}{dW} = \frac{K}{V} = k, \text{ whence } K = kV \tag{2}$$

TABLE I.

Curie-power hours. (W)	$\dfrac{Q}{Q_0}$	$k = \dfrac{1}{W} \log_e \dfrac{Q_0}{Q}$ $k \times 10^2$
3.845	0.821	5.01
8.014	0.684	4.75
10.000	0.615	4.87
12.000	0.075	4.61
		Mean $k = 4.81 \times 10^{-2}$ a.d. $= \pm.16$

TABLE II.

Effect of Variation of the Volume of Enzyme Solution Irradiated.

Volume. (V)	Curie-power hours. (W)	$\dfrac{Q}{Q_0}$	$k = \dfrac{1}{W} \log_e \dfrac{Q_0}{Q}$ $k \times 10^2$	(kV) $K \times 10$
cc.				
4.59	9.000	0.544	6.77	3.11
8.12	9.000	0.678	4.33	3.51
11.88	9.000	0.771	2.90	3.45
18.34	9.000	0.832	2.05	3.76
			Mean $K = 3.46 \times 10^{-7}$ a.d. $= \pm.18$	

where V is the volume of solution irradiated, and K is a constant.   In Tables II and III the results of two such experiments are given,[10]

[10] For these experiments spherical glass bulbs were made. The volume of the bulb was determined by making a pipette to deliver a volume of water sufficient to just fill the container when the emanation bulb was placed in position. The pipette was then calibrated by weight with distilled water.

where the values obtained for K are shown. The agreement obtained between these values appears to be significant.

In the experiments described above the average power of the radioactive source lay between 100 and 550 millicurie-powers.

TABLE III.

Effect of Variation of the Volume of Enzyme Solution Irradiated.

Volume. (V)	Curie-power hours. (W)	$\dfrac{Q}{Q_0}$	$k = \dfrac{1}{W} \log_e \dfrac{Q_0}{Q}$ $k \times 10^2$	(kV) $K \times 10$
cc.				
4.59	5.089	0.708	6.82	3.13
8.12	9.000	0.653	4.74	3.85
11.88	13.17	0.681	2.93	3.48
18.34	20.34	0.650	2.11	3.87
			Mean K = 3.58 $\times$ 10^{-1} a.d. = $\pm$.28	

CONCLUSION.

The radiochemical inactivation of invertase by beta radiation from the radioactive products in equilibrium with radium emanation can be explained quantitatively on the same basis as that of trypsin and pepsin previously reported; namely, the rate of change in the logarithm of the concentration of the active enzyme with respect to the variable, W, is constant, under the conditions of irradiation described, when the volume of solution exposed is constant. When, within the limits stated in this paper, this volume (V) is varied, the rate of radiochemical change is inversely proportional to V; i.e.,

$$-\frac{d \log Q}{dW} = k = \frac{K}{V}$$

THE EFFECT OF RADIATIONS FROM A MERCURY ARC IN QUARTZ ON ENZYMES.

I. The Effect of Ultra-Violet Radiation on Pepsin in Solution.

By RAYMOND G. HUSSEY and WILLIAM R. THOMPSON.

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In several communications we have described the results of experiments in which we observed the effect of the radiations (beta and gamma) from the radioactive products in equilibrium with radium emanation[1] on certain enzymes in dilute solution, namely trypsin,[2] pepsin,[3,4] and invertase.[5]   In each instance we found that the enzyme was inactivated, and that the form of the curve representing the course of inactivation was the same.   In the case of trypsin we investigated the effect of x-rays also,[2] and observed that definite inactivation of the enzyme resulted.   However, owing to the conditions of irradiation with x-rays, we were unable to study the kinetics of the reaction. More recently we have obtained some preliminary results from experiments wherein we have observed the effect of the radiations from a mercury arc in quartz on dilute solutions of pepsin.   In this communication we wish to present some of the results obtained.

Technical Procedure.

The pepsin solutions were prepared as follows: 17.50 gm. of granular pepsin were dissolved in distilled water containing 14.00 cc. of

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[1] We have obtained evidence which indicates that the inactivation is effected essentially by the beta radiation.[4]

[2] Hussey, R. G., and Thompson, W. R., *J. Gen. Physiol.*, 1922–23, v, 647.

[3] Hussey, R. G., and Thompson, W. R., *J. Gen. Physiol.*, 1923–24, vi, 1.

[4] Hussey, R. G., and Thompson, W. R., *J. Gen. Physiol.*, 1925–26, ix (in press).

[5] Hussey, R. G., and Thompson, W. R., *J. Gen. Physiol.*, 1925–26, ix, 211.

0.1 M HCl and diluted to 1 liter in a volumetric flask. The solution was filtered and a crystal of Merck's Reagent Thymol was added. The pH of this solution was 4.4 according to colorimetric determination. The solution was kept at 10°C. in a pyrex flask. The active pepsin present was measured by Northrop's viscosity method.[6] A volume of 4 cc. of the pepsin solution was irradiated.

The source of radiation was placed beneath a thermoregulated water bath (maintained at 10.0 ± 0.1°C.) in the bottom of which, directly above the center of the lamp, was set a polished circular quartz disc window (with an aperture 1 inch in diameter). The solutions to be irradiated were placed in a quartz tube of 1 inch diameter with a flat bottom in the thermostat in position 0.5 cm. above the quartz window, so that the opening of a shutter would throw a beam of radiation upon it. During irradiation the solution was stirred continuously by a mechanical device. The lamp was allowed to burn for at least 30 minutes before commencing an irradiation. Other conditions of irradiation were as follows: The distance between the lamp and the quartz window was 16.9 cm.; the thickness of the quartz window was ⅛ inch, and the thickness of the layer of water between this window and the bottom of the quartz tube containing the enzyme solution was 0.5 cm.

<div align="center">EXPERIMENTAL RESULTS.</div>

We found that the enzyme was inactivated by the radiations absorbed. Under certain conditions of irradiation the rate of change in the logarithm of the concentration of active enzyme with respect to time was found to be a constant, i.e.—

$$\frac{d \log Q}{dt} = - k \quad (1); \quad \text{or} \quad \frac{dQ}{dt} = - kQ \quad (2)$$

where Q represents the concentration of active pepsin expressed in arbitrary units, and t is time in hours.

<div align="center">DISCUSSION.</div>

It will be observed that the values for the velocity coefficient (k) given in Table I differ in the two experiments. Obviously the relation

[6] Northrop, J. H., and Hussey, R. G., *J. Gen. Physiol.*, 1922-23, v, 353.

between the quantities stated in equation (1) will hold only when the intensity of the radiation is constant and it is our opinion that this condition was not satisfied in the two experiments. Further investigation of this consideration is now in progress.

The results of the experiments reported appear to be sufficiently satisfactory to permit the statement that the effect of the radiation from a mercury arc in quartz is similar to the effect of the radiations from the radioactive products in equilibrium with radium emanation. This fact is of importance in connection with discussions regarding the mechanism of photochemical reactions.[7]

TABLE I.

Experiment.	Hrs.	Units of active pepsin found.	$k = \frac{1}{t} \log_e \frac{Q_0}{Q}$
		$Q_0 = 3.41$	
1	1.0	2.79	0.201
	2.0	2.02	0.262
	3.0	1.67	0.237
	4.0	1.24	0.253
2	1.0	2.85	0.181
	2.0	2.57	0.142
	3.0	2.06	0.166
	4.0	1.76	0.166

The value of Q_0 is the mean of 8 observations.
The value of Q found after irradiation is the mean of 4 observations.

CONCLUSION.

Pepsin in solution is inactivated by the radiations from a mercury arc in quartz. It would seem that the effective radiations are those in the ultra-violet region of the spectrum. The form of the curve describing the course of the inactivation is the same as that found for monomolecular chemical change.

[7] Bayliss, W. M., Principles of general physiology, London, 4th edition, 1924, 577.

X-RAY DIFFRACTION PATTERNS FROM PLANT FIBERS.

By O. L. SPONSLER.

(From the University of California, Southern Branch, Los Angeles.)

PLATE 3.

The finer structure of the cell-wall of plant fibers was anticipated years ago in the theories of Nägeli,[1] Strasburger,[2] and others, but the actual existence of a lattice arrangement of structural units was not definitely established until x-ray methods were applied to the problem. Several years ago x-ray diffraction patterns were obtained from plant fibers[3] and a space lattice was computed from the data thus made available.[4] In an attempt, by the author, to confirm the conclusions of the earlier investigators, Herzog and Jancke, certain discrepancies led to an intensive study of the diffraction patterns. A different method was used and more than twenty new lines were found on the photographs, while some of those previously reported were missing. This lack of agreement reopened the field for further investigation. (*Cf. Nature*, 1925, cxvi, 243.)

Method and Apparatus.

The method of obtaining the data upon which this report is based was very similar to that of Hull[5] for producing x-ray diffraction patterns from crystal powders, and the apparatus was practically the same as that described in many places in the literature. A quite detailed

[1] Nägeli, K., Über den innern Bau der vegetabilischen Zellenmembranen, 1864.

[2] Strasburger, E., Über den Bau und das Wachstum der Zellhäute, Jena, 1882, 225.

[3] Herzog, R. O., and Jancke, W., *Z. Physik*, 1920, iii, 196.

[4] Polanyi, M., *Z. Physik*, 1921, vii, 149. Herzog, R. O., *Cellulosechemie*, 1921, No. 8, 101.

[5] Hull, A. W., *Phys. Rev.*, 1917, x, 2nd series, 661; 1921, xvii, 2nd series, 571. Bain, E. C., *Chem. and Metallurg. Eng.*, 1921, xxv, 657; Davey, W. P., *Gen. Elec. Rev.*, 1922, xxv, 565.

description was given when reporting the work done on starch,[6] which will furnish the reader with information concerning the principles of x-ray reflection involved in this work, and with the method of interpreting diffraction lines in terms of a space lattice.

A standard Coolidge x-ray tube with a water-cooled molybdenum anticathode was the source of radiation. It was run at 42,000 volts, and the exposures were usually 25 milliampere-hours. A zirconium oxide screen made the beam practically monochromatic with a wave length of 0.71 Å. u., that of the K_α line. The cassette, or film holder, was much like that referred to in the earlier paper,[6] with slight modi-
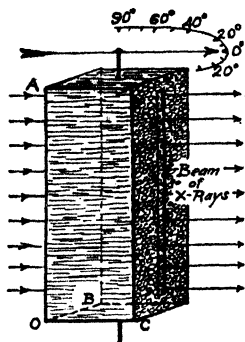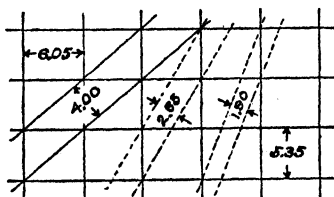
FIG. 1.   FIG. 2.

FIG. 1. Block of fibers with indicator needle attached and set at 0° position. Fibers parallel to OC. End view of fibers represented by small circles. The long, narrow rectangle represents a cross-section of the beam of x-rays, the path of which is indicated by the arrows.

fications made to allow for the rotation of a block of fibers. This block was built by laying thousands of fibers approximately parallel and compressing them into a tablet 3 mm. thick by 15 mm. wide, the length limited to that of the fibers. After squaring up the end of the tablet, a piece 3 mm. long was cut from it across the fibers, making a small block 3 × 3 × 15 mm. This small block (see Fig. 1) consisted of short lengths of fibers 3 mm. long, which were for the most part parallel to OC. The ends of the fibers appeared at the BOA face and at the face opposite.

[6] Sponsler, O. L., *Am. J. Bot.*, 1922, ix, 471; *J. Gen. Physiol.*, 1922–23, v, 757.

The block, with an indicator needle attached to it, was placed at the center point of the cassette, where it was adjusted in such a way that when the needle was set at 0° on the protractor the *OC* edge of the block, and therefore most of the fibers, were parallel to the line of propagation of the x-ray bec m. The beam, then, would pass lengthwise through most of the fibers. When the block was turned so that the needle pointed to 90°, the beam would pass through at right angles to the long axes of the fibers.

The diffraction patterns which were obtained from these two positions were quite different from each other. The pattern from the 0° position consisted of three prominent and three faint lines; that from the 90° position, of five or six strong lines and about as many weak ones.

Photographs were made from several kinds of fibers, but the lines obtained from ramie (*Bœhmeria nivea*) were used almost exclusively in this work because they were much more clean cut than those from either hemp (*Cannabis sativa*) or spruce (*Picea sitchensis*).

The lines on the photographs (Plate 3) indicate the existence of atoms or reflecting units of some kind arranged in sets of parallel planes, each line usually bearing a definite relation to a specific set of planes. The distance between the planes of a given set is computed from the formula $n\lambda = 2d \sin \theta$, where λ is the wave-length of the x-rays used, d the distance between the planes, and θ is the glancing angle determined from measurements of the lines on the photographs.[5-7]

The accuracy with which the spacing, d, could be determined was about 1 per cent. The blurring of some of the lines brought the error occasionally up to about 2 per cent, rarely more than that. For example: the 6.10 value was quite certain to $\pm$ 0.07, 3.98 to about $\pm$ 0.04, 2.58 to $\pm$ 0.02, and 1.70 to $\pm$ 0.01, the error becoming smaller with the smaller spacing values.

In the following paragraphs it is convenient to refer to the corresponding spacings, planes, and lines by using the same figures; thus, the planes which are spaced 3.98 Å.u. will be referred to as the 3.98 planes and the line they produce as the 3.98 line.

[7] Bragg, W. H., and Bragg, W. L., X-rays and crystal structure, London, 4th edition, 1924.

The photographs of some of the diffraction patterns are not readily reproduced in half-tone because considerable blackening is caused by a general scattering of the x-rays from certain positions of the block of fibers. From other positions the lines stand out clearly on the negative. The 90° position is an example of the latter while the 0° position is one from which the lines are blurred into the background between them (see Plate 3).

Each line was produced by a set of parallel planes in the wall of the fiber. In order to locate those planes with reference to some readily ascertained axis such as the long axis of the fiber, it is necessary to show that the orientation of the *fibers* in the block corresponds to the orientation of the *planes* which produced the lines. In other words, the data deal with the *planes*, while it is the *fiber as a whole* with which we have to work. These fibers (ramie) were only 0.07 mm. in diameter and very flexible since they were several cm. long. Since the length of the block was only 3 mm. along the *OC* edge it was obviously made up of pieces of these long fibers. In building up the block, absolute parallelism between the fibers was, of course, not obtainable; but by making a tablet with full length fibers first and then cutting the block (Fig. 1) from that, it is quite probable that most of the fibers were approximately parallel to one another and also to the *OC* edge of the block. The deviation of the fibers from true parallelism very probably corresponded to a fairly symmetrical frequency distribution. From a microscopic inspection of the block most of the fibers seemed to lie within a range of about 8° on each side of a mean. That is there were about as many fibers lying parallel to *OC* as there were at 2° to *OC* and approximately the same number at any given position up to about 8°, but rather suddenly the number dropped off so that a much smaller number were lying 10° to *OC*, only a few 15°, and scarcely any were found which were lying 20° to the *OC* edge of the block.

Diffraction Patterns from the 0° Position.

Returning now to the diffraction patterns, we find that when the indicator needle was set at 0° the photograph showed that 6.10, 5.40, and 3.98 planes were in proper positions to reflect their corresponding lines; when it was set at 2° the same lines appeared and their density was about the same as before; and when set at 4°, 6°, and 8° respec-

tively there was still very little if any difference in the density. At 10°, however, the lines were slightly less dense; at 15°, decidedly less dense; at 20° they were scarcely visible and at 25° there was no trace of them, for the short exposures given in taking this series of photographs. If now we compare these changes in density with the changes in the number of fibers throughout the same range of angles, as given in the paragraph above, we find a correspondence which indicates that the planes responsible for the diffraction lines were *parallel to the long axis of the fiber.*

There is another point which should be considered briefly here before our conclusions may be fully justified. It concerns the glancing angle of the various planes. It is evident from the preceding paragraphs that the block of fibers could not act as a single large crystal, and that at any given angle between the 0° and the 20° positions there must occur a group of fibers in which planes, for example the 6.10, would be oriented properly for reflection of their line. The number of active fibers varied inversely with the angle, but not in direct proportion variation. If our assumption is correct that the long axis and the planes were parallel, then with the block set at 0° the 6.10 line would be produced by a group of fibers which were lying 3° to the OC line, since that is the glancing angle for the 6.10 planes; the 5.40 line would be produced by another group of fibers which were lying about 4°, and the 3.98 line by still another group slightly over 5° to the OC line. Theoretically there would be more fibers in position to reflect the 6.10 line than the 3.98, and further there would be more fibers active in reflecting the 6.10 line at the 0° position than, for example, at 4°. The difference in number would be small, relatively, in both cases, and the density of the lines would not be noticeably affected at ordinary exposures, but by careful adjustment of the exposures it was possible to show a direct correspondence between the diffraction line at its densest and the fibers which were parallel to OC, with a greater degree of accuracy than was brought out in the preceding paragraphs. We were able to show that the 3.98 planes were parallel to the long axis to within an experimental error of 2°; the 6.10 and 5.40, within an error of 4°.

The correctness of the assumption that these three sets of planes were all parallel to the long axis of the fiber then seems highly probable,

and if this is so they must bear very definite relations to one another. These relations are brought out graphically in Fig. 2. By trial it was found that when 6.05 planes form a 90° angle with 5.35 planes, the diagonals, as shown by the solid diagonal lines in Fig. 2, are spaced 4.00. In the figure the planes are represented as perpendicular to the paper. Their spacings agree with the observed values within the limits of experimental error. Both observed and calculated values are presented in Table I for comparison.

The agreement seems to be sufficiently close to warrant our acceptance of one view of the elementary cell as a rectangle 6.10 Å.u. by 5.40 Å.u.

TABLE I.

Interplanar Spacing. 0° Position of Fiber Block.

Observed.	Calculated.	Density of lines.
Å.u.	Å.u.	
6.10	6.05	Strong.
5.40	5.35	Medium.
3.98	4.00	Very strong.
2.65	2.65	Weak.
1.98	2.00	Very weak, second order.
1.93	1.90	" "

The orientation of these rectangles on a cross-section of a fiber is suggested by data from two sources. The fiber is a minute hollow cylindrical tube with walls about 0.02 mm. thick. The work of Strasburger[8] and of W. L. Balls[9] has demonstrated that the cellulose substance is deposited by the protoplasm of the cell, layer upon layer, on the inside of the wall, forming more or less concentric cylinders. If we assume the 6.10 dimension to be the distance between these concentric cylinders, there would be about 40,000 of them in a fiber whose wall is 0.02 mm. thick. The 5.40 dimension would be the distance between radial planes. In the diagram in Figs. 3 and 4 a cross-section

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[8] Strasburger, E., Über den Bau und das Wachstum der Zellhäute, Jena, 1882, Plate 3, Fig. 22.

[9] Balls, W. L., Development and properties of raw cotton, London, 1915, 73. Also *Proc. Roy. Soc. London, Series B*, 1919, xc, 543.

of a fiber is represented.   The wall is MN in thickness.   The several
sets of rectangles represent one view of as many groups of elementary
cells, enormously out of proportion to the rest of the diagram.   In
them the 6.10 planes are tangential to the fiber, the 5.40 planes are
radial.   The long axis of the fiber and the planes are perpendicular
to the paper.   If one imagines a flat beam of x-rays to pass length-
wise through this cylinder, at an angle of 3° to the long axis, it will be
seen that there are only two regions, AA, from which the 6.10 planes
will reflect to produce a 6.10 line.   If now the angle is changed from
3° to about 4°, that is to the glancing angle for the 5.40 planes, a 5.40
line would be reflected from two—and only two—other regions, BB,
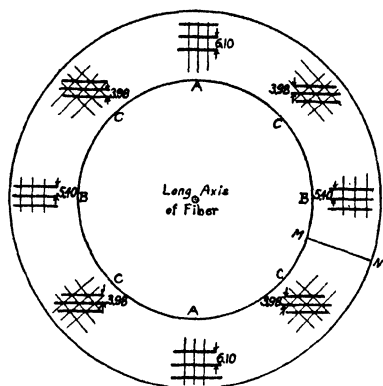
FIG. 3. Diagram of cross-section of fiber.   FIG. 4.
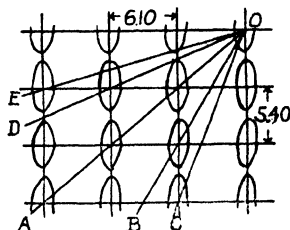
of the fiber.   If again the angle is changed to slightly more than 5°
the 3.98 planes will be in position to reflect a line, but then there will
be four regions $CCCC$ effective.   If all three sets of planes are not too
greatly different in reflecting power, the 3.98 line, since it is the result
of reflection from four regions instead of two, should be stronger than
either the 6.10 or the 5.40.   On the photographs it is decidedly the
densest, and to that extent there seems to be agreement with the
assumed cylindrical arrangement of the layers.

It seems possible that some conception of the units which make up
these layers may be gained from the diffraction pattern we are con-
sidering.   The spacings, 6.10, 5.40, and 3.98 are greater than would be
likely to occur if only single atoms were associated with the intersec-

tions.   It seems more probable that on the cross-section view of a fiber, as in Fig. 3, a group of atoms is centered around each intersection, since the atoms we are dealing with are about 1.5 Å.u. in diameter or less.   Also, it seems quite probable that the group is not arranged around the intersection in a radially symmetrical manner since the 6.10 line is denser than the 5.40, but that more atoms are associated with the 6.10 plane than with the latter.   The effect of that arrangement may perhaps be more clearly pointed out in Fig. 4 where the conventional elliptical characters, centered at each intersection of the lines representing 6.10 and 5.40 planes, indicate oblong groups of atoms. Planes such as *OD* or *OE* would be quite likely to fail to produce diffraction lines because of the annulling effect of interleaved planes.

TABLE II.

From Fig. 4.	Calculated.	Observed.	Density.
	$\overset{\circ}{A}$.u.	$\overset{\circ}{A}$.u.	
	6.05	6.10	Strong.
	5.35	5.40	Medium.
OA	4.00	3.98	Very strong.
OB	2.65	2.65	Weak.
OC	1.90	1.93	"
OD	2.46	—	———
OE	1.73	—	———
OA	2.00	1.98	Second order.

Those represented by *OB* or *OC* might produce faint lines, if any.   In Table II the observed and calculated values are included for comparison with the lines predicted from Fig. 4.   The agreement makes the assumption seem worth further consideration when determining the lattice structure.

By way of summary we may say that the data up to this point seem to justify the conclusions that the structural units in the fiber are arranged in concentric layers around the long axis, and that in those layers they are located so that they occur also in radial planes.   There seems to be no way of determining whether it is the 6.10 or the 5.40 value that is associated with the concentric layers.   The use of 6.10 for that spacing, in the discussion, was merely accidental.   There was neither experimental nor theoretical basis for the choice.

Diffraction Patterns from the 90° Position.

When the block of fibers was turned so that the indicator needle pointed to 90°, a diffraction pattern was obtained which consisted of about a dozen lines. With the block in that position most of the fibers were oriented approximately at right angles to the path of the beam of x-rays. Any lines, therefore, which appeared on the photographic film were necessarily reflected from planes which were approximately at right angles to the long axis of the fiber. The more exact position of those planes with respect to the long axis and to the planes already considered was determined by the same method as that used at the 0° position. Five photographs were taken, with short expo-

TABLE III.

Diffraction Lines from 90° Position of Fiber Block.

Interplanar spacing.	Density of line.
$\overset{\circ}{A}.u.$	
5.15	Strong.
3.40	Medium.
2.58	Very strong.
2.03	Strong.
1.70	"
1.46	Very weak.
1.29	" "
1.14	Weak.

sures, at intervals of 2° on each side of the 90° position. From them it was possible to establish rather satisfactorily several things. The angles formed by the 2.58 planes with the 6.10, 5.40, and 3.98 planes, and therefore with the long axis of the fiber, were found to be 90° ± 2°. Four other sets of planes, although not so readily located because their lines are weaker and not so sharply marked, seemed to be parallel also to the 2.58 planes. Another set of photographs of longer exposures brought out three more very faint lines whose planes seemed to be parallel to the 2.58 planes. In all, there were eight sets of planes apparently parallel to one another and perpendicular to the long axis of the fiber. In Table III are given the interplanar spacing values for these transverse planes, and the density of the lines which they produced.

The 2.03 line seemed to be composed of several superimposed lines, which have not been satisfactorily resolved.   On some of the negatives the value was computed as 2.01, on others 2.04, depending upon the position of the block.

The four other prominent lines were readily measurable within the limits of experimental error.   The 2.58 line might be considered as the second order reflection of 5.15 planes, except for the very obvious discrepancy in the densities.   It seems more probable that there were planes interleaved half-way between the 5.15 planes, which were not equal to the latter in reflecting power, analogous to the situation in rock salt in which the (111) planes are alternately weak and strong reflecting planes.[7]   The result produced there, just as here between the 2.58 and the 5.15 is a weak first and strong so called second order reflection.   A similar situation seemed to exist between the 3.40 and the 1.70 planes.   The relationship which seemed to exist between the various members of this group of parallel planes will be brought out later when the lattice structure is developed.

In Plate 3 it will be seen that planes spaced 2.58 and 1.70 form prominent lines which are too dense, as explained above, to have been produced by reflections of higher orders.   The atoms of which the planes were composed are about 1.30 Å.u. and 1.50 Å.u. in diameter. It would seem fairly probable, then, that in a lengthwise direction of the fiber the atoms would be less segregated into groups than they seem to be in a tangential or radial transverse direction.   In other words, from a cross-section view of a fiber the structural units would appear as fairly well isolated groups of atoms, while on a longitudinal view of the fiber they would appear separated laterally, but more closely associated in a lengthwise direction, forming fairly continuous strings of atoms running lengthwise of the fiber.

Diffraction Patterns from Positions between 0° and 90°.

With structural unit groups so arranged that they lie in transverse planes and at the same time form longitudinal layers in the fiber, they must also form diagonal planes which would produce diffraction lines when the block of fibers is turned to various positions between 0° and 90°.

This prediction was very clearly verified by several series of photo-

graphs taken with the block set at 5° intervals in some cases and 10° intervals in others, between 0° and 90°. A bewildering number of lines was obtained.

When sorted out, each line was found to appear from several adjacent positions and usually the position of greatest density was readily determined. For example the 4.35 line appeared on the negatives from the 50°, 60°, and 70° positions. The rate of change in density indicated that its densest position was probably between 60° and 65°. A series of short exposures taken at 2° intervals showed the position of most intense reflection to be very close to 62°.

TABLE IV.

Diffraction Lines from Positions between 0° and 90°.

Position of fiber block.	Interplanar spacing and density of lines.
	$\overset{\circ}{A}.u.$
80°	2.01 m.; 1.94 vvw.; 1.69 w.; 1.44 vw.
70°	3.20 s.; 1.94 vvw.; 2.35 w.; 1.25 vw.; 1.10 vw.
60°	4.35 vs.; 2.17 s.; 2.35 w.; 1.82 vvw.; 2.97 s.; 2.62 s.; 1.10w.
50°	2.62 vs.; 2.17 s.; 1.82 vvw.; 3.95 vw.
40°	6.40 vw.; 3.10 vw.; 1.88 vw.; 3.95 vw.; 1.55 vw.
30°	2.93 vw.; 3.10 vw.; 1.88 vw.; 2.65 w.
20°	2.65 w.
10°	2.65 w.; 2.34 vvw.

vs., very strong; s., strong; m., medium; w., weak; vw., very weak; vvw., only a faint trace.

In Table IV are given the interplanar spacing, density of line, and position of the fiber block at which the line appeared at its densest, for all of the lines obtained between the 0° and 90° positions. No attempt was made to estimate the position closer than the interval reading, which in this case is 10°. Wherever a line seems to be equally dense in two adjacent positions it is recorded in two places.

By revolving the block on an *OA* axis (Fig. 1) every possible set of planes was brought into an effective position for producing a diffraction line. That becomes evident only when one recalls that the block does not act as a single large crystal, and that the thousands of cylindrical fibers are laid more or less parallel to one another and to the *OC* edge of the block. In order to verify this, other blocks were made with

the fibers running parallel to OA instead of OC, and as expected the only lines produced were those which had appeared in the former when set at $0°$.

SUMMARY.

The rather long discussion just given seemed necessary in order to establish certain points before attempting to develop the lattice structure and before working out the identity of the structural unit of the ramie fiber.

1. Certain planes, 6.10, 5.40, 3.98, etc., as given in Table I, run lengthwise of the fiber; that is, they are parallel to the long axis.

2. These planes are in agreement with the assumption that one set, either the 6.10 or the 5.40 is tangential to the fiber and forms concentric cylinders, with the long axis of the fiber as the long axis of the cylinders; the other set, either the 5.40 or the 6.10. cuts the former at right angles and therefore its planes are radial with respect to the fiber, theoretically all of them meeting at the long axis, as indicated in the cross-section of a fiber in Fig. 3.

3. Other planes, 5.15, 3.40, 2.58, etc., as given in Table III, are transverse planes which form right angles with the long axis and therefore with the planes of Table I.

4. All of the planes are composed of reflecting units, probably groups of atoms, located at the intersections of the planes. This being the case, other reflecting planes must occur at other angles to the long axis. This prediction is verified by the lines given in Table IV.

5. The structural units in the wall of the fiber thus form a space lattice, the elementary cell of which is an orthorhombic structure.

6. Comparatively little can be said as yet concerning the structural unit. The unit is very probably composed of a group of atoms which are more or less closely packed together. If the groups were visible they would appear, in a cross-section of a fiber, as closely packed groups of atoms, 6.10 Å.u. from center to center of groups in one direction, and 5.40 Å.u. at right angles to that. In a longitudinal section, however, they would appear less compact and might even lose the appearance of groups in forming long strings of atoms which would extend lengthwise of the fiber.

By establishing the positions of the planes in the wall of the fiber, as in Tables I, III, and IV, it would seem that all dimensions of the elementary cell, and the size and character of the structural unit, could be determined.   Work along these lines is now in progress.

EXPLANATION OF PLATE 3.

FIG. 1. X-ray diffraction patterns of ramie fibers. Figures opposite the lines indicate the spacing of the planes which produced them. Those at the bottom give the position of the indicator needle at which the photograph was taken.
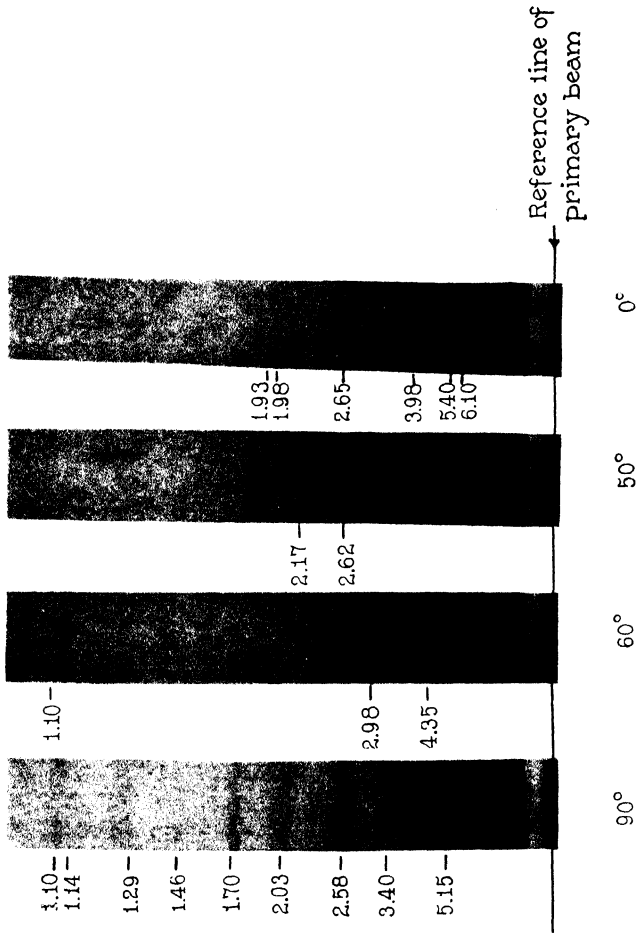
ACCUMULATION OF BRILLIANT CRESYL BLUE IN THE SAP OF LIVING CELLS OF NITELLA IN THE PRESENCE OF NH₃.

BY MARIAN IRWIN.*

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I.

INTRODUCTION.

It has been shown by several investigators[1] that when the pH value of the external dye solutions is increased, the rate of accumulation of a basic dye in the cell sap is accelerated. Two different explanations for this have been given. Some have accounted for it on the basis that the basic dye enters the cell in the form of a dye hydrate and combines with the substances in the cell; and that, therefore, the increase in the rate of accumulation of the dye is due to the increase in the concentration of the dye hydrate. Others[2] have assumed that the increase in the rate of accumulation of the dye is due primarily to the increase in the concentration of the combining substances in the cell caused by the increase in the pH value of the cell contents. From these standpoints it is of decided interest to see what will happen if we increase the pH value of the cell sap, while keeping that of the external solution constant. An investigation of this sort was made by McCutcheon and Lucke,[3] but from their

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* This work was done in part while the writer held a fellowship in the biological sciences, National Research Council, Washington, D. C.

[1] Overton, E., *Jahrb. wissensch. Bot.*, 1900, xliii, 669. Harvey, E. N., *J. Exp. Zool.*, 1911, x, 507. Robertson, T. B., *J. Biol. Chem.*, 1908, iv, 1. McCutcheon, M., and Lucke, B., *J. Gen. Physiol.*, 1923–24, vi, 501.

[2] Bethe, A., *Biochem. Z.*, 1922, cxxvii, 18. For other recent papers on the influence of pH on vital staining see Rohde, K., *Arch. ges. Physiol.*, 1920, clxxxii, 114. Pohle, E., *Deutsch. med. Woch.*, 1921, xlvii, 1464. Collander, R., *Jahrb. wissensch. Bot.*, 1921, lx, 354. Irwin, M., *J. Gen. Physiol.*, 1922–23, v, 223, 727.

[3] McCutcheon, M., and Lucke, B., *J. Gen. Physiol.*, 1923–24, vi, 501.

experiments it is not possible to draw a definite conclusion.[4]   They placed living cells of *Nitella*[5] in dye solutions at pH 8, containing in one case NH_4OH and in another case NaOH, and found that the dye accumulated in the sap less rapidly in the former than in the latter. Since the pH value of the cell sap increased in NH_4OH solution (without dye) while it did not change from the normal in NaOH solution (without dye) they concluded that the decrease in the rate of accumulation of the dye in the sap was due to the increase in the pH value of the sap, and that therefore the dye entered in the form of a dye hydrate, DOH, and combined with weak acids in the sap.   This conclusion, however, does not seem to be entirely justified for the following reason.   The change in the pH value of the sap is merely an indication that NH_3 (for convenience the term NH_3 will be used in this paper to represent aqueous NH_3 which includes undissociated ammonium compounds and NH_4 ions), has combined with substances in the sap, thus decreasing their power to combine with the dye when it enters.   In other words, there is a competition between the dye and NH_3 for the substances in the sap and it is quite possible that the competition may exist without noticeable increase in the pH value of the sap.   If there is sufficient buffer action, a considerable amount of NH_3 might accumulate without raising the pH value, but this would diminish the rate of accumulation of the dye since the NH_3 would compete with the dye for the substances in the cell.   The presence of such a competition does not necessarily mean that the dye enters the cell in the form of DOH, and that the combining substances are weak acids.   In case the dye salt, DCl, unites with the salts of proteins or of weak acids, the rate of accumulation of the dye will be decreased when NH_3 is present in the cell, provided the affinity of NH_3 for the substances in question is greater than the affinity of the dye for the same substances.   The reaction might be of the ordinary type where NH_3 combines with proteins or weak acids to form salts which are ionized and which can combine with the dye or NH_3 might combine with a salt to form a compound which

[4] *Cf.* Irwin, M., *J. Gen. Physiol.*, 1925–26, viii, 147.
[5] The same results were obtained by them with *Gonionemus* and starfish eggs. See Foot-note 3.

cannot combine with the dye, similar to the compound[6] formed by the union of copper hydrate with sodium tartrate, with which sodium hydroxide cannot react.

The possibility must also be borne in mind that NH_3 in the external dye solution may hinder the dye from entering the cell. McCutcheon and Lucke concluded, on the ground of experiments with the effect of NH_3 on the partition of dye between oil of sweet almonds and water, that the presence of NH_3 had no effect upon the taking up of the dye by the oil, but this may not necessarily prove to be the case with living cells.

In order to determine the cause of the decrease in the rate[7] of accumulation of dye in the presence of NH_3 it is desirable to carry out experiments which will show the rate of accumulation of the dye in the sap; (1) when the pH values of the sap are the same, while the concentrations of NH_3 in the sap are varied, which will show if there is a competition between NH_3 and the dye in the sap without a change in the pH value of the sap; (2) when the pH values of the sap and the concentrations of NH_3 in the sap are the same while the concentrations of NH_3 in the external solutions are varied, which will show if the presence of NH_3 in the dye solution can hinder the penetration of the dye into the cell; and (3) when the pH values of the sap are varied, while the concentrations of NH_3 in the sap remain practically constant, which will show if an increase in the pH value of the sap alone can bring about a decrease in the rate of accumulation of the dye.

[6] Norris, J. F., The principles of organic chemistry, New York, 1912, 272.

[7] It is evident that the competition of NH_3 would affect both the rate of accumulation of the dye in the sap and the concentration of the dye in the sap at equilibrium. In this paper the rate alone is studied since the cells die before the final equilibrium is attained. The rates are taken as near the beginning as possible and represent the concentrations of the dye in the sap at a given time in all cases. It is assumed that such rates would be approximately proportional to the concentrations of dye in the sap at equilibrium, if the reaction were not complicated by secondary processes as described in the writer's previous paper (see Foot-note 4).

II.

Accumulation of Brilliant Cresyl Blue in the Sap When Living Cells Are Placed in a Solution of Dye Containing NH_4Cl.

In order to see how much decrease occurs in the rate of accumulation of the dye in the sap when the cells are placed in a solution of dye containing NH_4Cl, cells were divided into three lots. The
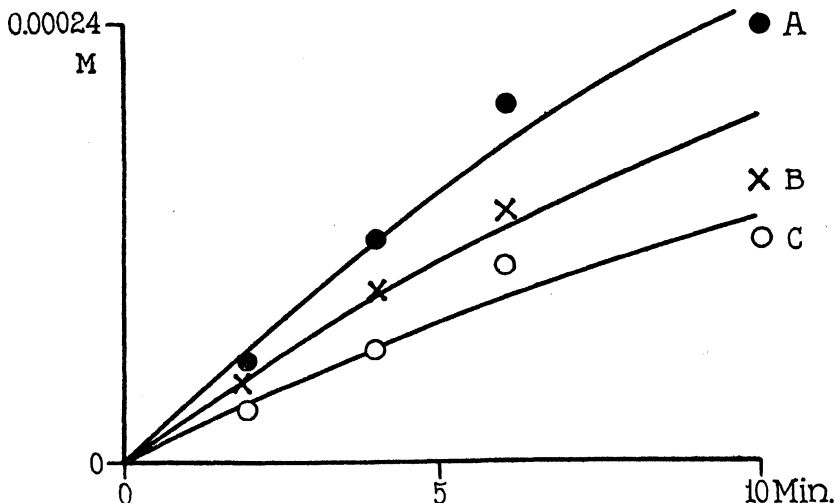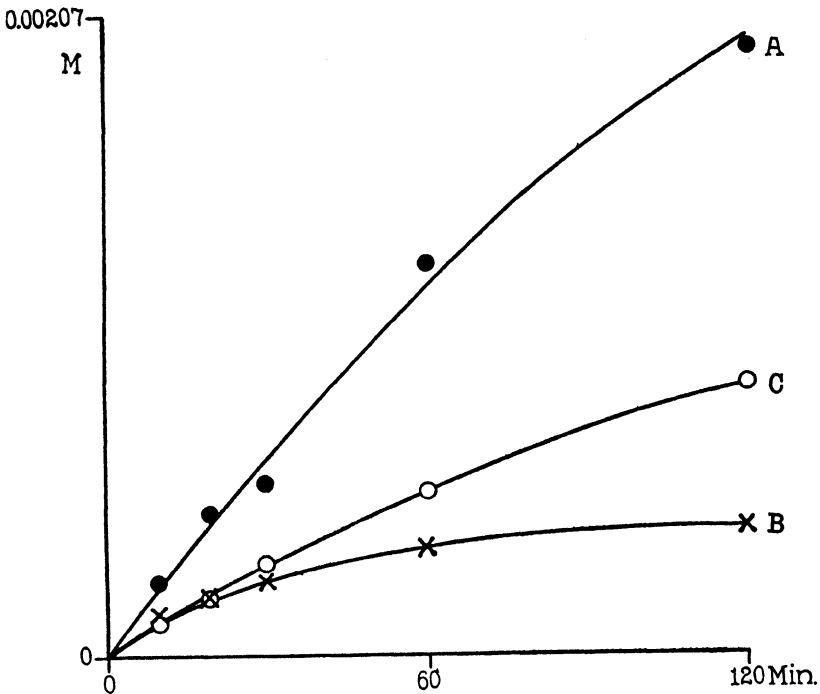

FIG. 1. Curves showing the rate of accumulation of dye in the sap of living cells of *Nitella*. The ordinates represent the concentrations of dye in the sap, and the abscissæ represent time. Curve A shows the rate of accumulation in 0.00014 M dye solution at pH 6.9; Curve B shows the rate in 0.00014 M dye solution at pH 6.9 containing 0.005 M NH_4Cl; Curve C shows the rate in 0.00014 M dye solution at pH 6.7. Each point on every curve is an average of forty experiments and the probable error of the mean is less than 7 per cent of the mean.

first lot was placed in 0.00014 M dye solution at pH 6.9, the second lot in the same concentration of dye solution at the same pH containing 0.005 M NH_4Cl, and the third lot in the same concentration of dye solution at pH 6.7. The concentration of the solutions was kept constant. All solutions were made up with $\dfrac{M}{150}$ phosphate buffer mixtures.[8] The experiments were made at 25 $\pm$ 0.5°C.

[8] The writer wishes to thank Mr. E. S. Harris for determining the pH values of the solutions by means of the hydrogen electrode.

At definite intervals, a few cells were removed, wiped, and the end of each cell was cut and the sap gently squeezed out on a glass slide. The sap was then drawn up into a capillary tube, and the color of the

FIG. 2. Curves showing the rate of accumulation of dye in the sap of living cells of *Nitella*. The ordinates represent the concentrations of dye in the sap, and the abscissæ represent time. Curve A shows the rate of accumulation in 0.00014 M dye solution at pH 6.9; Curve B shows the rate in 0.00014 M dye solution at pH 6.9 containing 0.005 M NH_4Cl; Curve C shows the rate in 0.00014 M dye solution at pH 6.7. Each point on every curve is an average of forty experiments and the probable error of the mean is less than 7 per cent of the mean.

tube was matched[9] with that of a tube of the same diameter containing a known concentration of the dye. The results are shown in Figs. 1 and 2.

On comparing the rate of accumulation of the dye in these three

[9] For details of technique see the writer's previous paper, *J. Gen. Physiol.*, 1925–26, viii, 147. In all the figures the curves are drawn free-hand through the points to give an approximate fit.

different dye solutions, it is found that in the first lot the rate is the highest from the start, while in the second lot the rate is higher at the start than in the third, but it becomes lower after about 25 minutes, as shown in Figs. 1 and 2, Curves A, B, and C. These curves indicate clearly that when the cells are placed in a solution of dye containing NH₄Cl, the rate of accumulation of the dye in the sap falls off from the start and this decrease becomes greater as the time goes on.

<p style="text-align:center">III.</p>

Change in the pH Values of the Cell Sap.

In order to carry out such experiments as are discussed in the introduction it is first of all necessary to obtain time curves showing the changes in the pH values of the cell sap when living cells are placed in solutions with[10] and without NH_3.

The pH of the sap was determined by the colorimetric method as follows: A capillary tube was filled for 1 inch with the sap; another capillary tube of the same diameter was filled for $1/10$ of an inch with about 0.005 per cent brom-cresol purple. The contents of the two tubes were then mixed on a glass slide and the entire amount of mixed sap and indicator was drawn up into a third capillary tube the color of which was carefully matched with that of a fourth tube having the same diameter as the third and filled with a standard phosphate buffer solution of known pH value containing the same concentration of the indicator as the third tube. The sap contains about 0.1 M halides so that the salt error should be corrected to obtain absolute values, but since the importance of these experiments lies in the relative values, this correction was omitted.

The solutions of NH₄Cl were made up in $\dfrac{M}{150}$ phosphate buffer mixtures at pH 6.9, and the concentration was kept constant throughout the experiment. The experiments were made at $25 \pm 0.5°C$.

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[10] Hoagland, Davis, McCutcheon, Lucke, and the writer have found that the increase in the pH value of the cell sap takes place when cells are placed in a solution containing NH_3. See Foot-notes 3 and 4, and also, Hoagland, D. R., and Davis, A. R., *J. Gen. Physiol.*, 1922–23, v, 629.

When the cells were placed in 0.005 M NH₄Cl solution at pH 6.9 and the change in the pH value of the sap was followed at definite intervals until death took place, it was found that after 5 minutes the pH value of the sap began to increase and in about half an hour it changed from pH 5.6 (normal) to pH 5.94 beyond which there was very little change in the pH value as shown in Fig. 3, Curve A, until almost all the cells were dead (within 6 hours after they were placed in the solution).
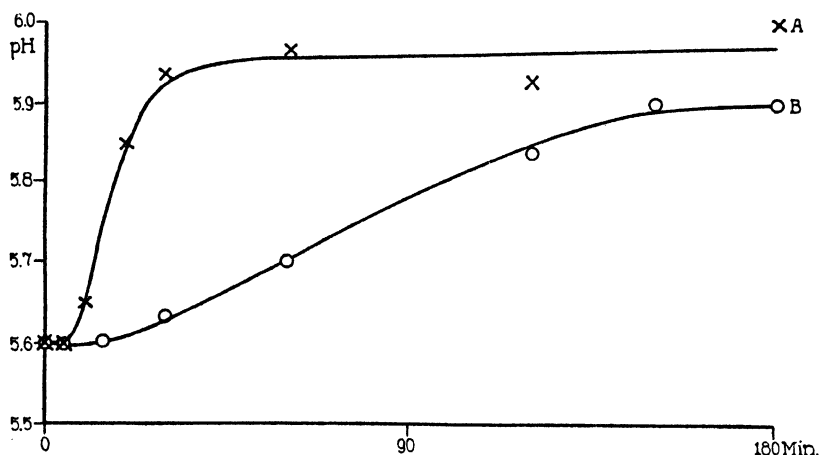
FIG. 3. Time curves showing the change in the pH value of the sap of living cells of *Nitella*. The ordinates represent the pH values of the sap, and the abscissæ represent time. Curve A shows the changes in the pH value when the cells are placed in 0.005 M NH₄Cl at pH 6.9. Curve B shows the changes in the pH values when the cells are placed in boric acid and sodium hydrate mixtures at pH 10.1. Each point on every curve is an average of forty experiments and the probable error of the mean is less than 7 per cent of the mean.

When living cells were placed in a buffer solution at pH 10.1 $\left(\dfrac{M}{40}\right.$ boric acid + NaOH), and the pH value of the sap was determined at intervals, it was found that the pH value began to change in about 20 minutes and in 2½ hours increased from 5.6 to 5.9, after which there was a very little change as shown in Fig. 3, Curve B, until almost all the cells were dead (in about 5 hours).

On comparing Curves A and B in Fig. 3, it is found that the pH value of the sap changes more rapidly, and the flattening of the curve is reached more quickly in the case of the cells placed in 0.005 M NH_4Cl solution at pH 6.9 $\left(\dfrac{M}{150} \text{ phosphates}\right)$ than in the case of the cells placed in pH 10.1 $\left(\dfrac{M}{40} \text{ boric acid } + \text{ NaOH}\right)$ without NH_4Cl.

If we base our notion of injury to the cells on the rapidity of death in the solution, then there is a very little difference between the cells placed in these two solutions, in that the cells in 0.005 M NH_4Cl solution at pH 6.9 die in about 6 hours while the cells placed in pH 10.1 solution without NH_4Cl die in about 5 hours. But if we base the criterion of injury[11] on the power of recovery there is a considerable difference as shown by the following experiment. When the cells that had been placed in 0.005 M NH_4Cl solution for 1 hour and other cells that had been in pH 10.1 buffer solution for $2\frac{1}{2}$ hours were removed from the solutions, wiped, and placed in distilled water (in which the cells normally live for days), almost all the former cells were found living after 24 hours, while the latter were almost all dead in 2 hours. In all probability cells become more or less injured when the pH value of the sap is appreciably changed but the injury to the cells under these conditions in NH_4Cl solution is much less and the recovery is more apt to occur than when the cells are placed in NaOH plus boric acid buffer solution. This injury increases and the cells die if left in these solutions for several hours. It is not possible to change the pH values of the cell sap in solutions containing NH_3 or NaOH in such a way that one can definitely say that the cells are not injured at the time the pH value of the cell sap is altered. The conditions described above are the most favorable which the writer has been able to find.

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[11] If we base the criterion of injury in the appearance of masses of chlorophyll in the sap, the observation is not accurate, though in general we may state that there are greater masses of chlorophyll in the sap of an injured cell than in the sap of a normal cell.

IV.

Accumulation of NH₃ in the Sap When Living Cells Are Placed in NH₄Cl Solution.

Since the experiments in Section III show that when the cells are placed in NH_4Cl solution the pH value of the sap increases progressively until an apparent equilibrium is established, it is desirable to see what type of curve is followed in the accumulation of NH_3 in the sap.

Cells were placed in 0.005 M NH_4Cl solution at pH 6.9 $\left(\dfrac{M}{150}\right.$ phosphates$\left.\right)$ at $25 \pm 0.5°C$. The concentration of the solution was kept constant. The concentration of NH_3 in the sap was determined by means of Nessler's reagent in the following manner. A few cells were removed from the solution and wiped with a wet cloth (free from NH_3). The ends of the cells were cut and the sap was gently squeezed out on a glass slide. Then the sap was drawn up into a capillary tube (about 10 inches in length), until it filled the tube for the distance of 1 inch. The sap was then blown into the Nessler tube containing 50 cc. of distilled water and 1 cc. of the Nessler reagent. The solution was then carefully shaken. A standard solution was made by taking the same amount (as in the case of the sap) of a known concentration of NH_4Cl solution and putting this into a Nessler tube containing 50 cc. of distilled water and 1 cc. of the Nessler reagent in the same manner as in the case of the sap. The colors of the two tubes were then compared by looking into the solutions from the top of the tubes. Since the color of the solution deepened on standing in both cases, it was necessary to make determinations at a definite time after the solutions were made up. For the present purpose the color was matched immediately.

The sap is not a clear liquid like the standard solution but it contains a viscous substance, which rises to the top of the tube and makes the readings difficult so that an accurate determination of an absolute value of NH_3 is not possible but this did not interfere with the present experiments since we needed only such relative values as could be obtained by this method.

The distilled water did not contain a measurable amount of NH_3 so that this was considered to be at a zero concentration for the sake of comparison since this distilled water was used for the Nessler test.

Tests showed that NH_3 adhering to the surface was not sufficient
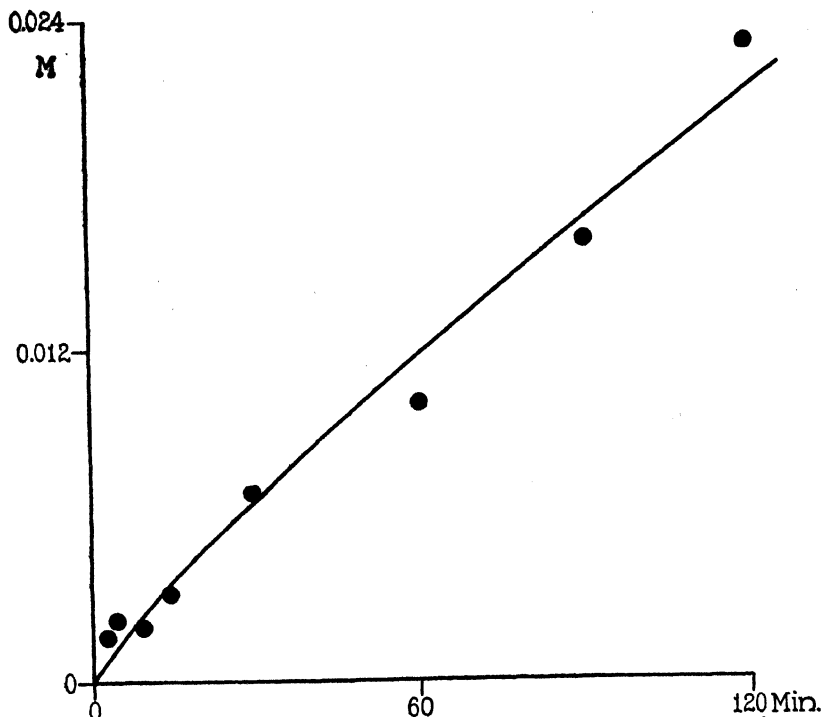
FIG. 4. Curve showing the rate of accumulation of NH_3 in the sap when living cells of *Nitella* are placed in 0.005 M NH_4Cl at pH 6.9. The ordinates represent the concentrations of NH_3 in the sap and the abscissæ represent time. Each point on the curve is an average of forty experiments and the probable error of the mean is less than 7 per cent of the mean.

to bring about noticeable errors. When the cells were dipped for a second in 0.005 M NH_4Cl and then wiped, and the sap was examined for NH_3 as described above, it was found that the sap gave no test for NH_3.

When living cells were placed in 0.005 M NH_4Cl at pH 6.9 and a few were removed at definite intervals for the determination of NH_3

in the sap, it was found that the accumulation of NH_3 took place gradually in the sap without reaching an equilibrium before the cells died (in about 6 hours). See Fig. 4.

When the relation between the concentration of NH_3 in the sap and the extent of the change in the pH value of the sap is considered,
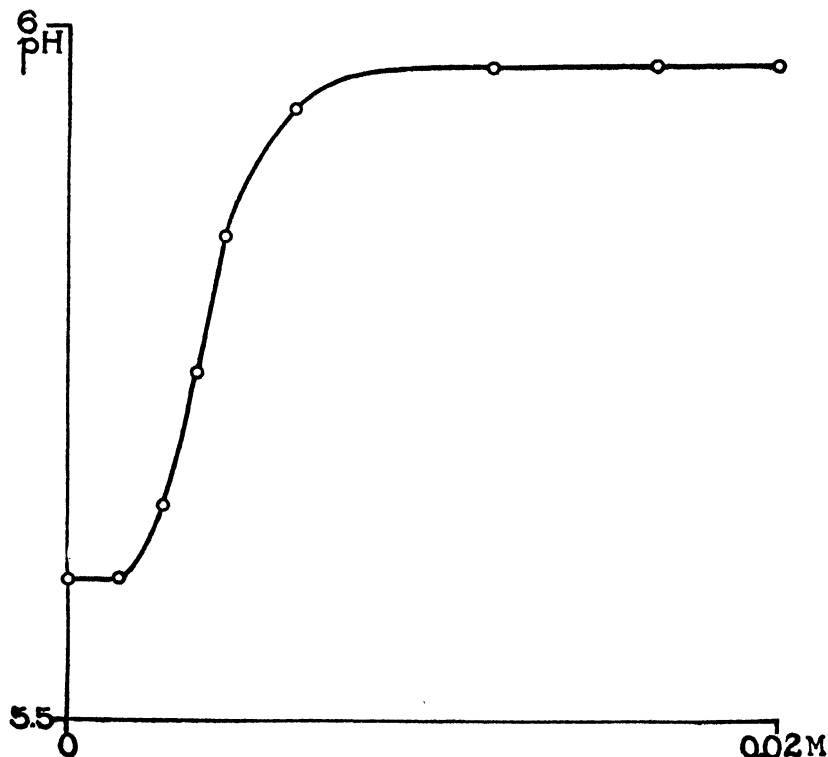
FIG. 5. Curve showing the relation between the concentration of NH_3 in the sap and the pH value of the sap. The ordinates represent the pH values and the abscissæ represent the concentrations of NH_3. The points on the curve are obtained from the curves as drawn in Fig. 3 (Curve A) and in Fig. 4.

the following is found to be true, as shown in Fig. 5. At the start the pH value of the sap remains unchanged until the concentration of NH_3 in the sap has reached 0.0014 M; this may be regarded as due to the buffer action of the sap (provided it is not NH_4Cl which enters the cell). Above this concentration the pH of the sap

increases but when the concentration of NH_3 in the sap has reached about 0.0064 M further accumulation of NH_3 brings about no appreciable change in the pH value of the sap until the cells die.

V.

Accumulation of the Dye in the Sap When the pH Values of the Sap Remain Constant While the Concentrations of NH_3 Are Varied.

When Figs. 3 and 4 are compared it is seen that after the cells have been placed in 0.005 M NH_4Cl solution at pH 6.9 $\left(\dfrac{M}{150}\right.$ phosphates$\left.\right)$ the accumulation of NH_3 in the sap takes place for about 5 minutes without a measurable change in the pH value of the sap. This enables us to carry out experiments in which we can compare the rate of accumulation of the dye in two lots of cells, one having no NH_3, and the other having 0.0014 M NH_3 in the sap (NH_3 is found to remain[12] in the sap during the experiment) while the pH value of the sap is the same in both cases.

Cells were placed in 0.005 M NH_4Cl solution at pH 6.9$\left(\dfrac{M}{150}\right.$ phosphates$\left.\right)$ for 5 minutes after which they were removed, wiped, and placed in 0.00014 M dye solution at pH 6.7 $\left(\dfrac{M}{150}\right.$ phosphates$\left.\right)$, at 25 ± 0.5°C. and the rate of accumulation of the dye was measured at different intervals for 6 minutes. It was found that the rate (Fig. 6, Curve B) was slightly lower than that obtained in the case of the cells dipped for a few seconds in NH_4Cl and placed in the same dye solution. (Fig. 6, Curve A.) The decrease is about 24 per cent which was about the extent of decrease found at the end of 5 minutes when the cells were placed in 0.00014 M dye solution at pH 6.9 containing 0.005 M NH_4Cl solution (Fig. 1, Curves A and B, in Section II). This indicates that the decrease is not due to the fact that NH_3 in the external dye solution hinders the entrance of the dye, but it was due to the presence of NH_3 in the sap. Since these

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[12] On removing the cells (containing 0.0014 M NH_3 in the sap) from 0.00014 M dye solution at pH 6.9, after 6 minutes, and testing the sap for NH_3, it is found that the NH_3 has not come out at all.

experiments were carried out when the cells had been placed in the NH$_4$Cl solution for only 5 minutes, we may assume that the cells were not injured at this stage.

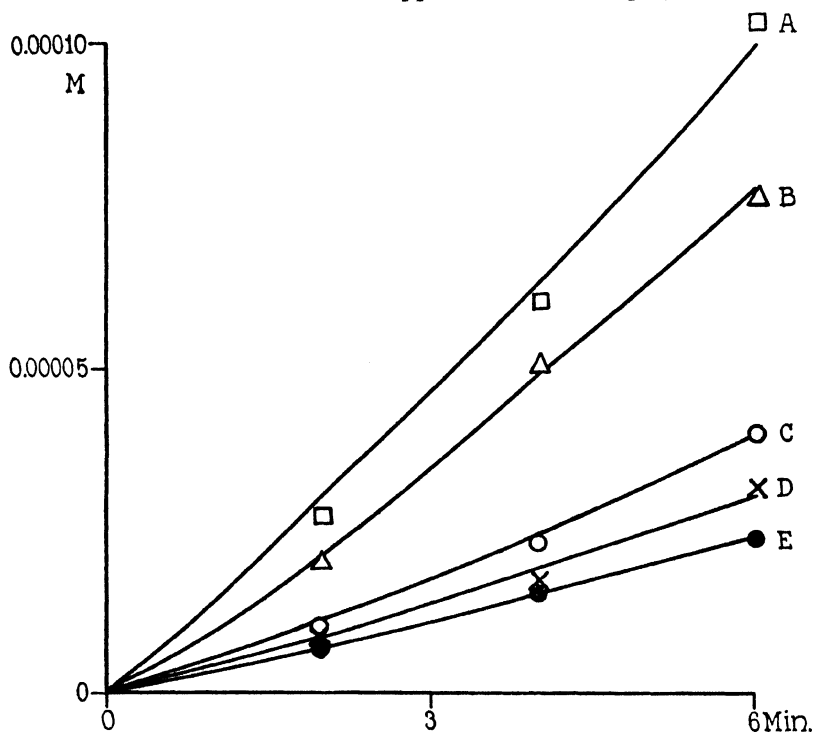It is of interest to see what happens when we employ cells whose

FIG. 6. Curves showing the rate of accumulation of dye in the sap of living cells of *Nitella* when the cells are placed in 0.00014 M dye solution at pH 6.7 after the cells have been treated for different lengths of time in 0.005 M NH$_4$Cl at pH 6.9. Curve A shows the rate when cells have been dipped for a few seconds in NH$_4$Cl; Curve B for 5 minutes; Curve C for 30 minutes; Curve D for 60 minutes, and Curve E for 120 minutes. Each point on every curve is an average of forty experiments and the probable error of the mean is less than 7 per cent of the mean.

sap has increased in alkalinity after a longer exposure, such as may possibly produce injury. The experiments in Sections III and IV show that the pH value of the sap is about 5.95, when the cells have been placed in 0.005 M NH$_4$Cl solution at pH 6.9 either for 30 min-

utes, 60 minutes, or 120 minutes though the concentration of NH_3 in the sap in the first case is 0.0064 M, in the second it is 0.0117 M, and in the third it is 0.0214 M. Such cells were removed from the NH_4Cl solution after 30, 60, and 120 minutes, wiped, and placed in 0.00014 M dye solutions at pH 6.7 $\left(\frac{M}{150} \text{ phosphates} \right)$ at 25 ± 0.5°C. When the rate of accumulation was measured at different intervals during 6 minutes (in this interval the concentration of NH_3 in the sap remained unchanged),[13] it was found that the rate decreased as the concentration of NH_3 increased in the sap as shown in Fig. 6, Curves C, D, and E. The rate of accumulation of the dye in the sap decreased about 24 per cent when there was 0.0014 M NH_3 in the sap, and when the pH of the sap was found to be 5.6 (normal value). There was about 62 per cent decrease in the case of the cells which contained 0.0064 M NH_3 in the sap (pH value of sap was about 5.93). There was a decrease of about 71 per cent when the cells contained 0.0117 M NH_3 in the sap (pH value of the sap about 5.97). At this concentration of NH_3 in the sap the extent of decrease seemed to have almost reached its maximum since in the case of the cells containing 0.0214 M NH_3 in the sap at pH 5.97 there was only about 76 per cent decrease. This seems to indicate that the effect of NH_3 on the rate of accumulation of the dye in the sap reaches a maximum at a definite concentration of NH_3.

VI.

Further Experiments to Ascertain If NH_3 in the External Solution Hinders the Entrance of the Dye.

It has been indicated by the experiments described in Section V that NH_3 does not hinder the entrance of the dye unless NH_3 penetrates into the sap, but in order to confirm this the following experiments were made. When the cells have been placed in 0.005 M NH_4Cl at pH 6.9 $\left(\frac{M}{150} \text{ phosphates} \right)$ for 1 hour it is found that

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[13] When cells containing 0.0064 M, 0.0117 M, or 0.0214 M NH_3 in the sap are placed in 0.00014 M dye solution at pH 6.7 for 6 minutes, then removed, and the NH_3 of the sap determined, it is found that no NH_3 has come out of the sap.

0.0117 M NH$_3$ has accumulated in the sap; at this stage there is no measurable increase in NH$_3$ in the sap if the cells are left in the solution 6 minutes longer.

Cells were therefore placed in the NH$_4$Cl solution for 1 hour, removed, and wiped. One lot was now placed in 0.00014 M dye at pH
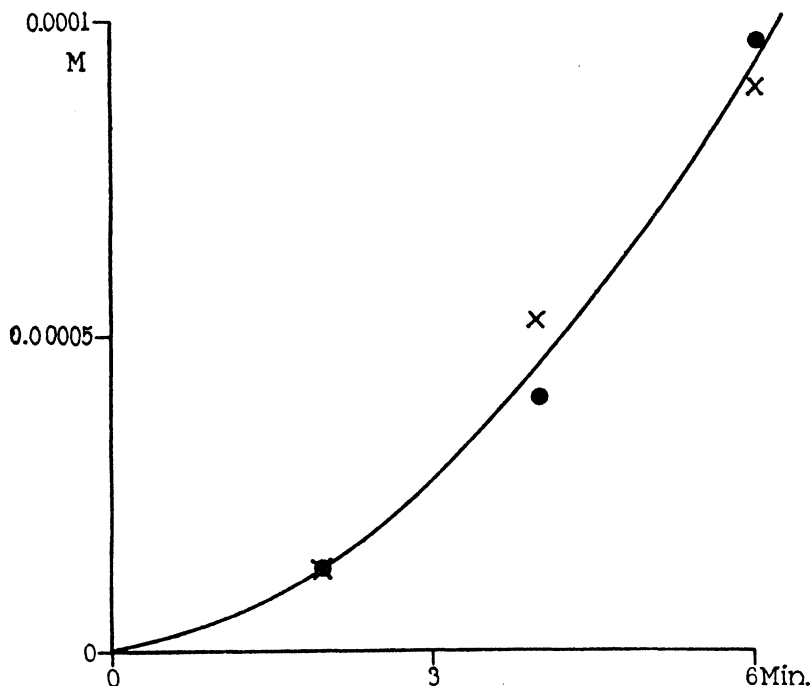

FIG. 7. Curves showing the rate of accumulation of dye in the sap of living cells of *Nitella* when cells are placed in a solution of dye after they have been placed for 1 hour in 0.005 M NH$_4$Cl at pH 6.9. The symbol ($\times$) represents t he rate of accumulation in 0.00014 M dye solution at pH 6.9, while the symbol ($\bullet$) represents the rate in 0.00014 M dye solution at pH 6.9 containing 0.005 M NH$_4$Cl. Each point on every curve is an average of forty experiments and the probable error of the mean is less than 7 per cent of the mean.

6.7 $\left(\dfrac{M}{150} \text{ phosphates}\right)$ and another lot in the dye solution of the same concentration and the same pH value containing 0.005 M NH$_4$Cl at 25 $\pm$ 0.5°C. When the rate of accumulation of the dye in the sap was measured at intervals during 6 minutes, it was found that

the rate was the same in both cases as shown in Fig. 7, symbol ●
and symbol ✕. Thus it is evident that it is only the NH_3 in the sap
which affects the accumulation of dye and that so long as this is
constant variations to the above extent in the concentration of
NH_3 in the external solution are of no import.

<div align="center">VII.</div>

*Accumulation of the Dye in the Sap When the pH Values of the Sap
Are Varied and the Sap Contains No NH_3.*

Since it is evident from the experiments described above that the
presence of NH_3 in the cell sap brings about a decrease in the rate of
accumulation of the dye, the next step is to ascertain whether the
increase in the pH value of the cell sap (in absence of NH_3) will have
the same effect. Although we cannot obtain cells having the same
concentrations of NH_3 in the sap, while the pH values of the sap are
different, it is possible to change the pH values of the sap by placing
the cells in solutions at pH 10.1 $\left(\dfrac{M}{40} \text{ boric acid} + \text{NaOH mixtures.}\right)$
Fig. 3, Curve B shows that in this mixture the pH value of the sap
increases but little in 30 minutes. This increase continues until
the pH of the sap is changed from pH 5.6 to 5.9 in $2\dfrac{1}{2}$ hours after
which there is very little change until the cells are dead.

Cells were placed in this mixture at $25 \pm 0.5°C$. for 5 seconds,
15, 30, 60, and 150 minutes, removed from the solutions, wiped, and
placed in the 0.00014 M dye solution[14] at pH 6.7 $\left(\dfrac{M}{150} \text{ phosphates}\right)$
at $25 \pm 0.5°C$. An exposure of 15 minutes to the buffer solution at
pH 10.1 caused a noticeable decrease in the subsequent accumulation

[14] It is not possible to determine the pH value of the cell sap after the dye
has entered the sap, hence another experiment was made. When the cells which
had been in pH 10.1, as described in the text, for different lengths of time, were
removed, and placed in pH 6.7 $\left(\dfrac{M}{150} \text{ phosphates}\right)$ at 25°C. for 6 minutes, it was
found that the pH values remained the same. From this result it is assumed
that the pH values of the sap will not change when cells are placed in the dye
solution at pH 6.7 for 6 minutes.

of the dye and this decrease became greater the longer the cells were left in the solution at pH 10.1 (Fig. 8).
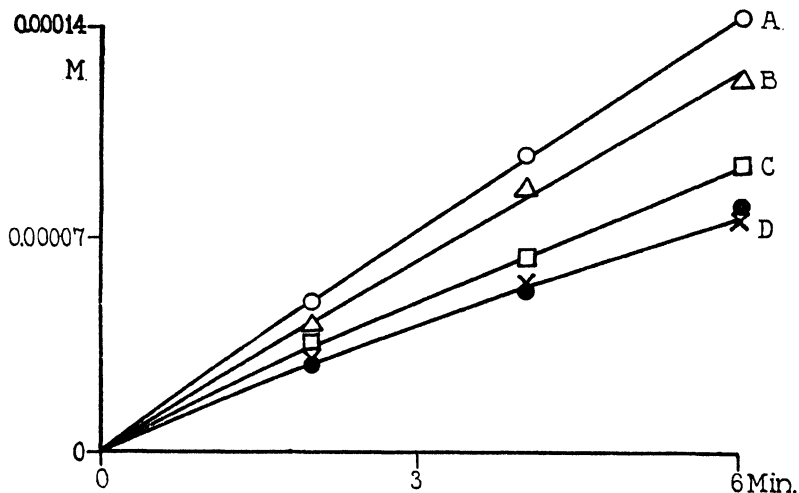

FIG. 8. Curves showing the rate of accumulation of dye in the sap of living cells of *Nitella* which have been placed in $\frac{M}{40}$ boric acid and sodium hydrate mixtures at pH 10.1 and then removed and placed in 0.00014 M dye solution at pH 6.7: Curve A, in the buffer solution for a few seconds; Curve B for 15 minutes; Curve C for 30 minutes; Curve D for 60 minutes (×) and 150 minutes (●). Each point on every curve is an average of forty experiments and the probable error of the mean is less than 7 per cent of the mean.

Fig. 8 shows that there may be a decrease of 13 per cent in the rate[15] of accumulation of the dye in the sap though the pH value of

[15] After the cells had been dipped in the $\frac{M}{40}$ buffer mixture of boric acid and sodium hydroxide at pH 10.1, wiped, and placed in 0.00014 M dye solution at pH 6.7 as already described, the rate of accumulation of the dye was greater than that found in the case of the cells which had been exposed in the same way to a buffer solution at pH 6.7 $\left(\frac{M}{150} \text{ phosphates}\right)$. When such cells were left for 2 minutes in the phosphate solution the rate was the same as in unwashed cells. Whether this increase is due to a preliminary stage of an injury, or to an adhering of the buffer mixtures to the surface of the cell, which cannot be readily washed out, and which produces an effect of the buffer mixtures on the surface of the cell apart from injury, cannot be stated definitely at present.

the cell sap remains normal (pH 5.6). There is a decrease of about 35 per cent when the pH value of the sap increases from 5.6 to 5.63. It is a striking fact that the decrease is only 44 per cent when the pH value of the sap reaches 5.7 or 5.9. From this it is evident that the decrease[16] in the rate may take place even when there is no change in the pH value of the sap, and that the extent of decrease reaches a maximum value when the pH of the sap is at 5.7. This may be due either (1) to the presence of substances, as in the case of NH_3, which compete with the dye for the combining substances in the sap, or (2) to injury to some part of the surface of the cell, which partly prevents the accumulation of the dye in the sap by allowing some to diffuse out of the cell, or (3) possibly to a combination of both.

VIII.

DISCUSSION.

The experiments described in the present paper show that there is a decrease in the rate of accumulation of the dye in the sap when NH_3 is present in the sap but that the presence of NH_3 in the external solution alone has no such effect.

The fact that NH_3 when present only in the external solution does not affect the entrance of the dye would seem to indicate that at the concentrations of the solution used there is no antagonism between NH_4^+ ions and D^+ ions in the sense that they might hinder each other from entering the living cell, and that there can be no tautomeric change in the dye brought about by NH_4Cl which could

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[16] When cells which had been placed in the $\frac{M}{40}$ boric acid and sodium hydroxide mixture at pH 10.1 for 2½ hours were wiped, and placed in 0.005 M NH_4Cl at pH 6.9, 0.0007 M NH_3 was found to have accumulated in the sap in 5 minutes. This is much less than the concentration of NH_3 (0.0014 M) in the sap of a control cell placed directly in NH_4Cl solution at pH 6.9. When such cells were removed from the solution of NH_4Cl, wiped, and placed in 0.00014 M dye solution at pH 6.7 it was found that the rate of accumulation of the dye had decreased considerably as compared with cells which had been exposed to the buffer mixture for the same period but which had not been placed in NH_4Cl solution. The pH of the sap in both cases was about 5.9, so that the decrease was due to the presence of NH_3 in the sap.

produce such an effect. It is also evident that the dissociation of the dye is not affected by NH_4Cl, at the concentrations employed.

The decrease in the rate of accumulation of the dye in the sap may be interpreted as due to the fact that NH_3 and the dye compete for certain substances in the cell. The degree of competition as expressed by the decrease in the rate of accumulation of the dye may be dependent on the dissociation constants of the dye and of the NH_3 in the sap, and on the concentrations of these two substances. This is to be expected if we assume that the dye enters as DOH and, like NH_3, is capable of combining with weak acids and proteins in the sap. If the dye enters as a dye salt, e.g. DCl, and combines with a salt of a weak acid or of protein it may also be affected by the competition of NH_3 as already explained in the introduction.

Though it is not possible to determine experimentally at present, the same type of competition may exist in the protoplasm or in the surface membrane of the protoplasm, so that the assumption made in a previous paper[4] regarding the rôle of the surface membrane of the protoplasm may not be wrong.

It is evident from what has been said that it is not possible to determine experimentally the exact relation between the pH value of the sap and the decrease in the rate of accumulation which is found in the presence of NH_3.

Thus it is not yet possible to state definitely whether or not the dye enters the cell as the dye hydrate, but experiments are being carried on by the writer which may lead to a definite conclusion in the near future.

SUMMARY.

When the living cells of *Nitella* are placed in a solution of brilliant cresyl blue containing NH_4Cl, the rate of accumulation of the dye in the sap is found to be lower than when the cells are placed in a solution of dye containing no NH_4Cl and this may occur without any increase in the pH value of the cell sap. This decrease is found to be primarily due to the presence of NH_3 in the sap and seems not to exist where NH_3 is present only in the external solution at the concentration used.

THE PENETRATION OF CO_2 INTO LIVING PROTOPLASM.[*]

BY W. J. V. OSTERHOUT AND M. J. DORCAS.

(From The Rockefeller Institute for Medical Research.)

Experiments with H_2S on *Valonia*[1] indicate that under normal conditions it is chiefly if not exclusively undissociated molecules which enter the cell.[2] In view of the importance of this conclusion it seemed necessary to carry out comparable experiments with another substance. For this purpose CO_2 was chosen. It penetrates readily and is easily measured. Its importance in the organism renders its investigation particularly significant.

It might be supposed that the production of CO_2 by the cell would interfere with the measurements but it was found that under the conditions of the experiments this is so slight as to be negligible.

The experiments were carried out by the junior author, the methods being similar to those employed in the experiments on H_2S.[2] The CO_2, generated by the action of dilute HCl on Bermuda coral rock, passed through a column of cotton and bubbled through sea water until the latter had absorbed about one-fourth of its volume. The desired pH value was obtained by adding HCl or NaOH.

In each case 10 cells (the average volume of a cell being about 0.33 cc.) were placed in a bottle containing 125 cc. (with no air space above the solution) and tightly stoppered.

The process of penetration was practically complete in less than 2 hours but the cells were left for several hours in order to make sure that equilibrium had been attained. The solution was stirred during the exposure.

The sap was collected as described in the previous paper.[1] 1 cc. was used for each analysis. It was free from contamination by sea

[1] *Valonia macrophysa*, Kütz, collected at Bermuda.

[2] Osterhout, W. J. V., *J. Gen. Physiol.*, 1925–26, viii, 131.

water as shown by the absence of SO_4. Sea water contains sufficient SO_4 to give a visible precipitate with acidified $BaCl_2$ when the sea water is diluted one hundred times. The absence of SO_4 also indicates that no injury has occurred and this is borne out by the fact that if cells which had been exposed to the experimental treatment were returned to sea water and kept under ordinary laboratory conditions they lived indefinitely.

The temperature ranged between 20° and 22°C. but did not vary more than 1° during 'any one experiment. The temperature coefficient (12.5° to 22.5°C.) of penetration and outward diffusion for living and dead cells is very low (less than 1.1).[3]

A Van Slyke apparatus[4] was employed to determine the total CO_2.

The essential question is whether the total CO_2 in the sap corresponds to the undissociated fraction of the total CO_2 in the sea water or to the dissociated fraction. The total CO_2 includes the HCO_3^- and CO_3^- ions, the undissociated H_2CO_3, the undissociated carbonates and bicarbonates of all the metals present, the uncombined or free CO_2, and an unknown number of hydrates and complexes of any one or all of the above.

If the concentration of total CO_2 inside the cell depends on the outside concentration of one of these substances, and is independent of the concentration of others, we should expect the ratio of concentration inside to that outside to change when we alter the proportion of this particular outside substance. There is no obvious method of changing only one of these substances. Changing more than one will serve our purpose provided that we do not change in the same ratio a substance which penetrates and one which does not, in such a way as to make it impossible to decide which substance penetrates.

The most expedient way is to change the H^+ ion concentration. An increase in the H^+ ion concentration would decrease the proportion of HCO_3^- and CO_3^- ions, and of other substances which change with them, and would increase the proportion of undissociated H_2CO_3 and of free CO_2.

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[3] A low temperature coefficient for the absorption of pure gaseous CO_2 by solutions of K_2CO_3 was observed by Williamson, R. V., and Mathews, J. H., *Ind. and Eng. Chem.*, 1924, xvi, 1157.

[4] Van Slyke, D. D., *J. Biol. Chem.*, 1917, xxx, 347.

Since we may assume that the relation between uncombined CO_2 and undissociated H_2CO_3 is approximately constant, we may for the purpose of the present paper (where relative values alone are considered) use the term undissociated H_2CO_3 to include the uncombined CO_2 (whose concentration is probably much greater than that of the undissociated acid). This course will be followed as a matter of convenience.

In order to ascertain the concentration of undissociated H_2CO_3 (including free CO_2) in the sea water at various pH values various means may be used. The method employed by McClendon is to determine the partial pressure of the CO_2 in the gas phase in equilibrium with the sea water (total CO_2 being constant). At low pH values, where the total CO_2 is undissociated, this partial pressure will reach its maximum value; as the pH value increases and H_2CO_3 begins to dissociate this value will fall in approximate proportion, *e.g.* when the total CO_2 is 50 per cent dissociated, the partial pressure will fall to about 50 per cent of its maximum value. The values of the partial pressures as determined by McClendon[5] are shown in Fig. 1 by the symbol ($\triangle$).

A somewhat different method (which gives similar results) was employed in the present investigation by circulating gas through sea water (at various pH values) and through artificial sap[6] (containing no excess base). If the pH value of the artificial sap is low enough to prevent dissociation of H_2CO_3, the amount of total CO_2 which passes over into the artificial sap corresponds to the undissociated H_2CO_3 (including free CO_2) in the sea water. For this purpose an apparatus was employed which consisted of two flasks fitted with tubes and an aspirator bulb so that the gas could be circulated and the two liquids brought to equilibrium with the same gas phase. The total CO_2 of the sea water and of the artificial sap was then determined with the Van Slyke apparatus. It was found that the lower the pH value of the sea water the higher was the relative total CO_2 of the artificial sap as compared with that of the sea water; this was true down to pH

[5] McClendon, J. F., Gault, C. C., and Mulholland, S., *Carnegie Institution of Washington, Pub. 251*, 1917, 36. These values are for sea water of excess base 25 (at 20°C.).

[6] This was made by mixing 86.24 cc. of KCl 0.6 M with 15.08 cc. of NaCl 0.6 M; *cf.* Osterhout, W. J. V., *J. Gen. Physiol.*, 1922–23, v, 225.

3 below which lowering of the pH value produced no increase in the relative concentration in the artificial sap. The H_2CO_3 was therefore regarded as undissociated at pH values below 3. If the concentration of total CO_2 in the sea water is compared with that in the artificial sap, the latter is found to be about 16 per cent higher at pH values of 3 or lower, due to the fact that CO_2 is more soluble in the artificial sap. This is not surprising since CO_2 is less soluble in solutions containing sulfate.[7]

Let us suppose, for convenience, that the concentration of total CO_2 in the sea water is kept constant, for example at 100. Its concentration in the artificial sap (in equilibrium with the same gas phase) will be proportional to the concentration of undissociated H_2CO_3 (including free CO_2) in the sea water.[8] Thus if the pH of the artificial sap is low enough to prevent dissociation of H_2CO_3 its concentration of total CO_2 will be 116 when the total CO_2 in the sea water is undissociated; it will be 58 when the total CO_2 of the sea water is 50 per cent undissociated. Hence it is evident that we can find the percentage of undissociated H_2CO_3 (including free CO_2) in sea water by expressing the concentration of total CO_2 in the artificial sap as per cent of the total CO_2 in sea water and multiplying these figures by $100 \div 116$. Thus at pH 3 the per cent of undissociated H_2CO_3 (including free CO_2) in sea water is 100, at pH 5.8 it is 86 per cent, and so on.[9] The results are expressed in Fig. 1 by the symbol ($\times$).

These values may be compared with those which would be expected on a theoretical basis if the CO_2 were dissolved in distilled water. The undissociated H_2CO_3 (including free CO_2) expressed as per cent of total CO_2 may be calculated by means of the formula:[10]

$$\text{Per cent undissociated } H_2CO_3 \text{ (including free } CO_2) = \frac{100}{1 + \dfrac{K_1}{(H)} + \dfrac{K_1 K_2}{(H)^2}}$$

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[7] *Cf.* Hildebrand, J. H., Solubility, New York, 1924, 140.

[8] It is assumed that the apparent dissociation constant is the same in both.

[9] These determinations are approximate. It is probable that if care had been taken to keep the pH value of the artificial sap (at equilibrium) low enough to suppress ionization of H_2CO_3 in all cases there would be less irregularity.

[10] *Cf.* Michaelis, L., Die Wasserstoffionenkonzentration, 2nd edition, Berlin, 1922, 48.

in which $K_1 = 3.3 \times 10^{-7}$ and $K_2 = 6 \times 10^{-11}$. The values obtained by this calculation are expressed in Fig. 1 by the symbol ($\square$). The curve has the same general form as those already discussed but the latter are displaced somewhat to the left in the lower part. This is to be expected since E. J. Warburg[11] has shown that the presence of
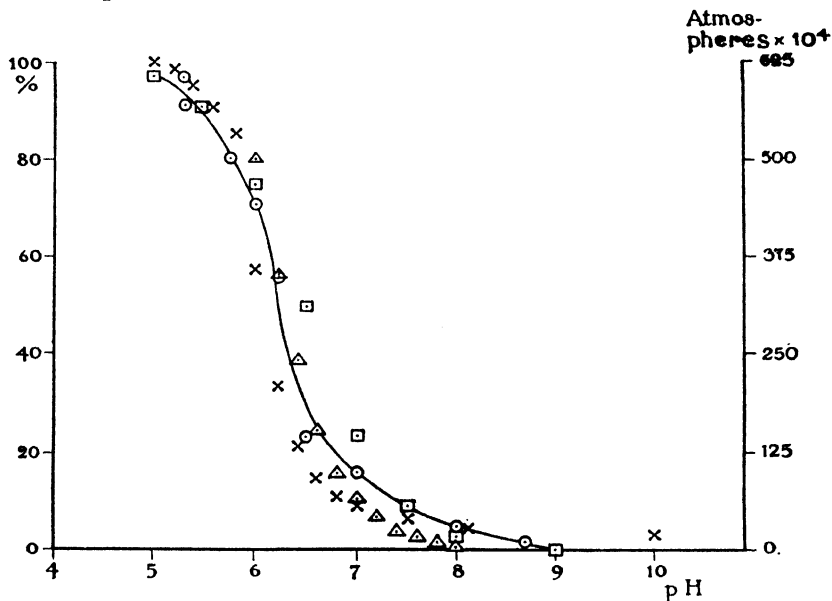

FIG. 1. Shows that the total CO_2 in the cell sap corresponds approximately to the undissociated H_2CO_3 (including free CO_2) in the sea water outside. The total CO_2 in the sap (o) is expressed as per cent of that in the sea water outside (the curve is drawn free-hand through the points to give an approximate fit). The per cent of undissociated H_2CO_3 (including free CO_2) as calculated from the dissociation constant is shown by the symbol ($\square$). The partial pressure of free CO_2 in the sea water as determined by McClendon is shown by the symbol ($\triangle$). It is expressed as ten thousandths of a standard atmosphere as shown by the figures on the ordinate at the right. The concentration of H_2CO_3 (including free CO_2) in sea water, expressed as per cent of that found at pH 3 (where the H_2CO_3 is regarded as undissociated), is shown by the symbol ($\times$): it may be regarded as expressing the per cent of total CO_2 which is in the form of H_2CO_3 (including free CO_2). Each experimental point of symbols ($\square$) and ($\times$) represents one determination.

[11] Warburg, E. J., *Biochem. J.*, 1922, xvi, 153. See also Van Slyke, D. D., Wu, H., and McLean, F. C., *J. Biol. Chem.*, 1923, lvi, 765.

salts lowers the value of the negative logarithm of the apparent first dissociation constant of H_2CO_3: that this would shift the curve to the left is obvious.[10]   The relation of this to the theory of Debye and Hückel has recently been discussed by Hastings and Sendroy.[12]

Let us now enquire what takes place inside the cell.   If CO_2 acts in the same general way as H_2S and enters the cell only in the form of undissociated molecules we should expect the concentration of total CO_2 in the cell to correspond to the concentration of undissociated H_2CO_3 (including free CO_2) in the sea water.   This expectation is realized as is evident from Fig. 1 in which the circles denote the concentration of total CO_2 found in the sap of living cells at various pH values after equilibrium is reached between the total CO_2 inside and that outside.   The curve is drawn free-hand through these points to give an approximate fit.   In order to facilitate comparison the concentration of total CO_2 inside is expressed as per cent of that outside: by this method the correspondence (or lack of it) between the total CO_2 inside and the undissociated H_2CO_3 outside can be made most clearly evident.   If, for example, at a given pH value the undissociated H_2CO_3 (including free CO_2) in the sea water is about 50 per cent of the total CO_2 we shall expect the concentration of total CO_2 inside the living cell to be about 50 per cent of the total CO_2 in the sea water.   Hence the curve for per cent of undissociated H_2CO_3 (including free CO_2) in the sea water should approximately coincide with that for total CO_2 in the sap (expressed as per cent of undissociated H_2CO_3, including free CO_2, in the sea water).

Fig. 1 shows that the correspondence between the total CO_2 in the living cell ($\bigcirc$) and the undissociated H_2CO_3 (including free CO_2) as shown by the symbols ($\times$) and ($\triangle$) is fairly good.   It cannot be expected to be exact for a number of reasons.   In the first place, as already noted, CO_2 is more soluble in the sap than in the sea water.   In the second place, if we assume that H_2CO_3 penetrates freely but that its ions cannot pass in or out, it follows that if it partly dissociates after entering the cell the ions so formed will be trapped: more undissociated H_2CO_3 will move in, until the concentration of undissociated H_2CO_3 is the same inside and outside.   The total CO_2 inside, consist-

[12] Hastings, A. B., and Sendroy, J., Jr., *J. Biol. Chem.*, 1925, lxv, 445.

ing of undissociated H_2CO_3 (including free CO_2), plus ions, will therefore be greater than the undissociated H_2CO_3 (including free CO_2) outside. The amount of dissociation is not sufficient to make any great difference. If we regard the pH of the cell sap as approximately constant at 5.8,[13] we may, for purposes of calculation, take the per cent of dissociation calculated from the dissociation constant of CO_2 dissolved in distilled water; *i.e.*, about 19 per cent of the total CO_2. If the concentration of undissociated H_2CO_3 (including free CO_2) outside is 100 we should have the same concentration inside and this would be 81 per cent of the total CO_2 inside which would therefore amount to $(100 \div 81) 100 = 123.46$. The same relation would hold no matter what the outside pH or concentration of undissociated H_2CO_3 happened to be, *i.e.* we should always expect to find 23.46 per cent more total CO_2 inside than undissociated H_2CO_3 (including free CO_2) in the sea water outside. This excess will of course be less if the per cent of dissociation of H_2CO_3 in the sap is less.

Fig. 1 shows that at higher pH values there is more total CO_2 in the sap than undissociated H_2CO_3 (including free CO_2) in the sea water as calculated from the experimental data of McClendon and the writers. This would be expected on the grounds just mentioned (solubility and dissociation). At lower pH values, however, so much CO_2 might enter the cell as to lower the pH value, in which case the dissociation would be less and the total CO_2 of the sap would fall off somewhat. The curve indicates that this may be the case but at lower pH values the total CO_2 of the sap falls more than would be expected, becoming less than the undissociated H_2CO_3 (including free CO_2) of the sea water. For this no explanation is at present suggested.

Below pH 6.0 the curve for penetration becomes a little uncertain. The cells are soon injured at this pH and when they die the total CO_2 becomes the same inside and outside. Frequently we find that when the outside solution is at pH 5.8 or 6.0 the total CO_2 of the sap attains only 85 to 90 per cent of the outside concentration of total

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[13] The pH value of the sap varied but little except when lowered by the penetration of CO_2 and the difference in dissociation caused by this lowering may be neglected for our present purpose.

CO_2 even after standing 4 hours, as might be expected. Yet at other times we find practically 100 per cent with every evidence of normal condition of the cells. It is thought that this difference is not due to experimental error because there is no difficulty in getting consistent results with dead cells under these conditions.

In general it would seem that the concentration of total CO_2 inside the cell is approximately equal to the total undissociated H_2CO_3 (including free CO_2) outside. Possibly this would not be the case if the sap had much buffer action. The buffer action of the sap towards acids was found to be of a smaller order of magnitude than that of sea water. McClendon's excess base number[14] for this sea water was 24.8[15] for a typical sample and for the sap was less than 0.5.

Successive drops of 0.01 N HCl or $Ba(OH)_2$ were added to 1000 drops of sap, of distilled water, and of boiled KCl solution respectively, and the change in pH was measured by comparing with standard indicators. The sap has but little buffer action as is shown in Fig. 2. Its buffer action is of the same order of magnitude as that of a solution of KCl, of the same concentration of chloride from which most of the CO_2 had been driven off by boiling. According to the graph, 32 drops would change the sap from pH 8.0 to 6.2 and the sea water requires 200 drops for the same change.

We therefore seem justified in assuming that the total CO_2 inside the cell corresponds approximately to the total undissociated H_2CO_3 (including free CO_2) of the outside solution, and that the relations of the various ions in the sap are not complicated by the presence of any large amount of buffer.

If the facts found in the case of *Valonia* are generally valid we should expect that when the interior of any living cell is much more acid than the surrounding medium (the excess base being the same in both)

. [14] The excess base is determined by boiling off the CO_2 from the sea water and observing how much 0.01 N HCl must be added to 100 cc. of sea water to bring the pH back to the value it had before boiling: the number of cc. added is the excess base number. The excess base may be regarded as that part of the base which is not bound by strong acids and which in this case is largely bound by H_2CO_3. *Cf.* McClendon, J. F., Gault, C. C., and Mulholland, S., *Carnegie Institution of Washington, Pub. 251,* 1917, 31.

[15] *I.e.* excess base = 0.00198 M.

the internal concentration of total CO_2 will be less than the external, providing the cell does not manufacture CO_2 rapidly enough to overcome the difference which would naturally exist at equilibrium. For example, in the case of *Valonia* we find that the total CO_2 content of the sap from cells in normal sea water (pH 8.2) is always less than that of the surrounding sea water. A typical analysis of cells that had
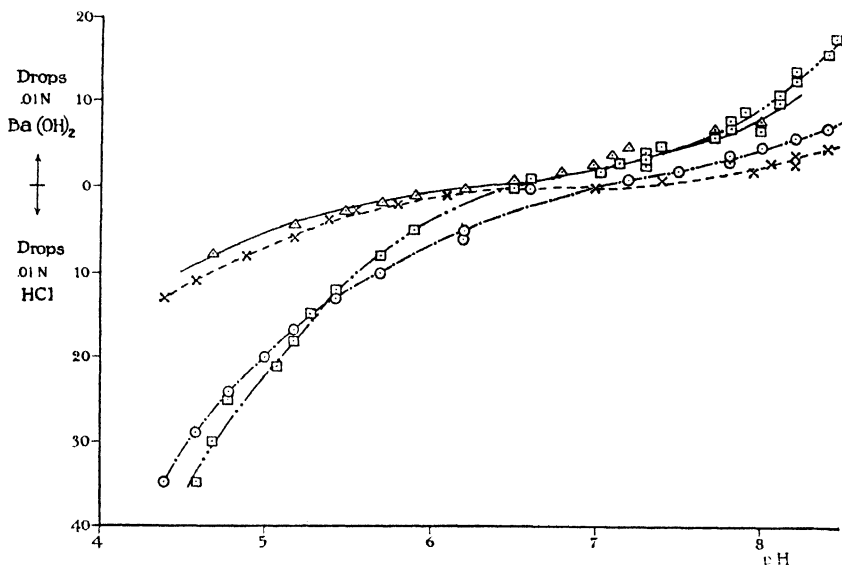

FIG. 2. Shows the buffer action of sap (□), of 0.6 M KCl (○), of boiled distilled water (×), and of unboiled distilled water from a wash bottle (△). The ordinates give the number of drops (of 0.01 N HCl or 0.01 N Ba(OH)₂) added to 1000 drops of the liquid. The curves are drawn free-hand to give an approximate fit. Each point represents one determination.

been 15 hours in a dark room showed that the sap had 0.009 cc. of total CO_2 per cc. of sap while the surrounding sea water had 0.038. A similar lot of cells after exposure to direct sunlight for 5 hours showed 0.004 cc. per cc. for the sap and 0.031 cc. per cc. for the sea water.

This has an important bearing on certain physiological problems in the study of which the assumption has frequently been made that the total CO_2 content of the cell at equilibrium is equal to that of the external medium.

Let us now consider another aspect of the penetration of CO_2 into *Valonia*. If we ignore the formation of $CO_3^=$ ions and assume that some indiffusible ions are present so that a Donnan equilibrium is set up with H^+ and HCO_3^- diffusing in and out freely, but not undissociated H_2CO_3 or CO_2 we might assume the relation

$$\frac{H^+ \text{ inside}}{H^+ \text{ outside}} = \frac{HCO_3^- \text{ outside}}{HCO_3^- \text{ inside}}.$$
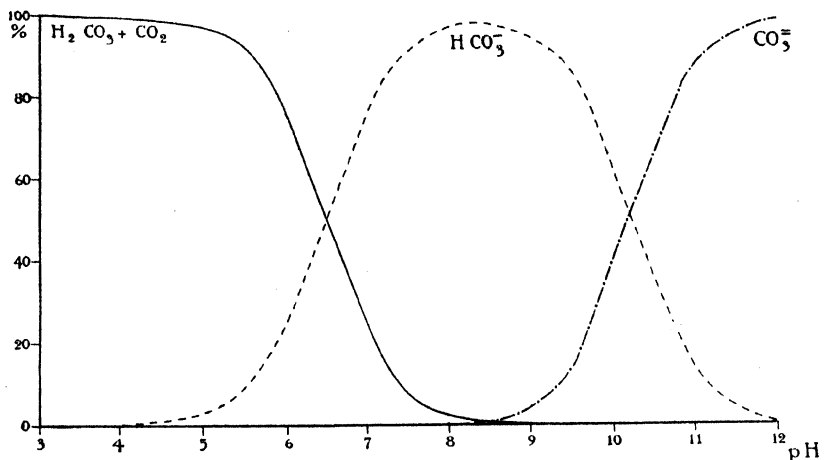

Fig. 3. Shows the per cent of undissociated H_2CO_3, including free CO_2, (———), of HCO_3^- (--------), and of $CO_3^=$ ($-\cdot-$) calculated from the formulæ:

$$\text{Per cent undissociated } H_2CO_3 \text{ (including free } CO_2) = \frac{100}{1 + \dfrac{K_1}{(H)} + \dfrac{K_1 K_2}{(H)^2}}$$

$$\text{Per cent } HCO_3^- = \frac{100}{1 + \dfrac{(H)}{K_1} + \dfrac{K_2}{(H)}}$$

$$\text{Per cent } CO_3^= = \frac{100}{1 + \dfrac{(H)}{K_2} + \dfrac{(H)^2}{K_1 K_2}}$$

in which $K_1 = 3.3 \times 10^{-7}$, and $K_2 = 6 \times 10^{-11}$.

All values are expressed as per cent of total CO_2.

Although this assumption may seem improbable,[16] it may be interesting to ascertain to what values it would lead.

If we regard the internal pH value as practically constant at pH 5.8 we should have, when the outside pH value is 6,

$$\frac{H^+ \text{ inside}}{H^+ \text{ outside}} = \frac{10^{-5.8}}{10^{-6}} = 1.59.$$

TABLE I.

A Comparison of $\dfrac{H^+ \text{ Inside}}{H^+ \text{ Outside}}$ *with* $\dfrac{HCO_3^- \text{ Outside}}{HCO_3^- \text{ Inside}}$ *at Various pH Values.*

It is assumed that the inside pH is constant at 5.8, and that $HCO_3^- = 19$ per cent of total CO_2 in sap.

pH inside.	pH outside.	Total CO_2 in sap.	HCO_3^- in sap.	HCO_3^- in sea water.	$\dfrac{H^+ \text{ inside}}{H^+ \text{ outside}}$	$\dfrac{HCO_3^- \text{ outside}}{HCO_3^- \text{ inside}}$
5.8	5.8	77	77(.19) = 14.63	19	$\dfrac{10^{5.8}}{10^{5.8}} = 1$	$\dfrac{19}{14.63} = 1.299$
5.8	6.0	71	71(.19) = 13.49	25	$\dfrac{10^6}{10^{5.8}} = 1.585$	$\dfrac{25}{13.49} = 1.85$
5.8	6.55	24	24(.19) = 4.56	53	$\dfrac{10^{6.55}}{10^{5.8}} = 5.63$	$\dfrac{53}{4.56} = 11.62$
5.8	6.95	17	17(.19) = 3.23	74	$\dfrac{10^{6.95}}{10^{5.8}} = 14.13$	$\dfrac{74}{4.07} = 22.9$
5.8	8.0	5	5(.19) = 0.950	97.3	$\dfrac{10^{8.0}}{10^{5.8}} = 158.5$	$\dfrac{97.3}{0.950} = 102.4$

From Fig. 1 we find that the total CO_2 in the sap at pH 6 = 71 (regarding the total CO_2 in the sea water as constant at 100). For the purpose of comparing the HCO_3^- in sap and sea water at the pH values here considered we may assume that all the CO_2 is H_2CO_3 (in ionized or non-ionized form) and consider that 19 per cent of the total CO_2 in the sap (Fig. 3) is ionized at pH 5.8: we then have as the ionized portion (71) (.19) = 13.49. From Fig. 3 we learn that all of this may

[16] Some objections to it have been stated in a previous paper.[2] The fact that the pH value of the sea water has been varied within wide limits (by adding HCl or NaOH) with little or no effect on the pH value of the sap indicates that H^+ ions do not diffuse in and out freely.

be regarded as HCO_3^-.   In the sea water we have at pH 6 (Fig. 3)
$HCO_3^- = 25$.   We therefore have

$$\frac{HCO_3^- \text{ outside}}{HCO_3^- \text{ inside}} = \frac{25}{13.49} = 1.85.$$

Proceeding in this manner we obtain the values given in Table I.
The figures in the last two columns increase in somewhat the same
fashion and the deviations are such as might result largely from experi-
mental errors.   The greatest discrepancy occurs where the total
CO_2 inside deviates most from the values calculated from the disso-
ciation constant (see Fig. 1).

It is evident that if it is the ions alone which enter, the rate of pene-
tration will increase as the pH value of the external solution increases,
while if it is only the undissociated molecules which enter, the rate of
penetration will increase as the pH value of the sea water falls.   The
latter is found to be the case, and this would indicate that it is the
undissociated molecules which enter unless for some reason the rate of
penetration is proportional, not to the concentration of the penetrating
substance in the sea water, but to its concentration at equilibrium in
the sap, which appears improbable.   It is of course possible that both
ions and undissociated molecules enter but that the latter penetrate
much more rapidly.

In order to arrive at the conclusion that ions enter, we are
obliged to make improbable assumptions, and unpublished electrical
experiments by L. R. Blinks make it difficult to believe that ions are
able to penetrate.   We may therefore conclude that little or no CO_2
enters except in the form of undissociated molecules.

In this connection it may be noted that the work of Loeb,[17] Harvey,

[17] Loeb, J., *Biochem. Z.*, 1909, xv, 255; 1910, xxiii, 95; *Arch. ges. Physiol.*,
1897–98, lxix, 1; 1898, lxxi, 457; Artificial parthenogenesis and fertilization,
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Physiol.*, 1924, lxviii, 183.   Smith, H. W., *Am. J. Physiol.*, 1925, lxxii, 347.

Crozier, Haas, Jacobs, M. M. Brooks (dealing with *Valonia*), Beerman, Clowes, Smith, and others, on CO_2 and on various weak acids, indicates that undissociated molecules penetrate, although the methods employed do not enable us to decide positively whether ions enter or not. Those who have concluded that ions do not enter have done so on indirect grounds. This is also true to some extent where the opposite conclusion has been reached (*cf.* Smith and Clowes, and Van Slyke, Wu, and McLean).[18]

SUMMARY.

The experiments indicate that little or no CO_2 enters normal cells of *Valonia* except in the form of undissociated molecules.

Whenever the interior of a cell is more acid than the surrounding medium (excess base being the same in both) we may expect that at equilibrium the internal concentration of total CO_2 will be less than the external.

[18] Smith, H. W., and Clowes, G. H. A., *Am. J. Physiol.*, 1924, lxviii, 183. Van Slyke, D. D., Wu, H., and McLean, F. C., *J. Biol. Chem.*, 1923, lvi, 765.

TEMPERATURE AND HEART RATE IN PTEROTRACHEA AND TIEDEMANNIA.

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I.

The attempt to give form and substance to various possibilities suggested by a study of thermal increments at present calls for an increase in the available data. It is necessary to analyze a greater array of biological processes in a greater variety of forms. Since the fauna at Naples affords abundant opportunities for investigations of this character, a number of organisms fairly common there were explored and, for the purposes in mind, the heart rates of the large heteropod *Pterotrachea coronata* and of the pteropod *Tiedemannia neapolitana* proved to be, in several respects, almost ideal. Aside from the great convenience of being completely transparent, both animals are very sensitive and permeable to heat, and their hearts are large enough to be seen clearly without a lens, under practically all circumstances. At intermediate temperatures pulsation in *Pterotrachea* in unit time is approximately twice as fast as in *Tiedemannia* where ten beats require slightly over 0.4 of a minute between 14° and 15°. Moreover, there are significant differences and resemblances between these two forms,—a fact which has acquired additional interest since Crozier and Stier (1924–25, *a*), have reported one

temperature characteristic for the heart rate of *Limax* ($\mu = 16,300$), and another for *Anodonta* ($\mu = 11,200$). With these values at hand, it is now possible to compare and interpret the increments for two other molluscs widely different in systematic affinities, in mode of life, and in physical consistency.

<div align="center">II.</div>

The observations on *Pterotrachea* were made after placing each animal in a separate aquarium, open to the air and containing about 1200 cc. of sea water. These vessels were stationed in a glass tank in which the level of the water was kept above that in the individual containers. To reach the upper ranges of temperature, definite amounts of water were removed from both the tanks and the aquaria and carefully replaced by equal quantities at higher temperatures. In this way both the levels and the differences of level were held sufficiently constant. The lower temperature limits were reached by allowing small flasks filled with ice, and corked, to float on the surface of the water in the containers, and by adding cold water or ice to the outer water jacket. In this case also, precautions were taken to maintain the original levels.

Final readings were begun at the lowest limits and on increasing the temperature 5 minutes were usually sufficient to establish the new equilibrium. In bringing this about, the uniform distribution of heat in the inner chambers was greatly facilitated by the gentle rhythm of the dorsal "fin" of the mollusc and moderate undulating movements on the part of the entire animal. *Pterotrachea* is almost never completely quiescent. The thermometers, like the animals, were always entirely submerged and after a given temperature had been maintained within 0.1–0.2° for 5 minutes, the time required for ten complete cycles of the heart was determined with a stop-watch. In the earlier observations this process was repeated five times for each temperature, but later only three groups of ten beats each were counted. The results derived in this way from eight individuals and covering a range, collectively, from about 4–27°, are plotted in mass in Fig. 1 according to Crozier's method (1924–1925, *a*) and the familiar Arrhenius equation (1915).

It is at once apparent that individuals must differ decidedly in the actual rate of the heart beat at any given temperature; also, that within definite limits, both vertical and lateral on the plot, the observations may be represented by a straight line. The thermal increment that best describes the points between $1/T = 0.00340$ and $1/T = 0.00350$, is 11,200. Above and below these regions other conditions plainly hold.
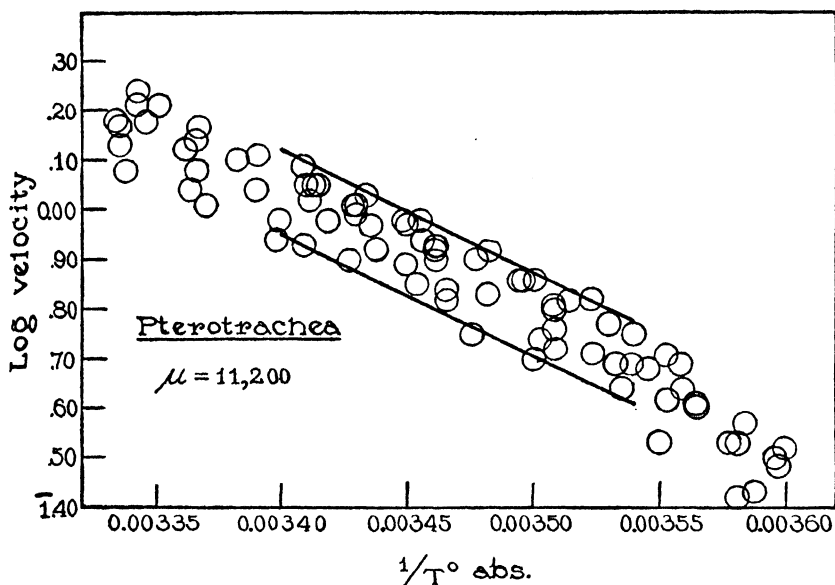

FIG. 1. Velocity of heart beat in *Pterotrachea coronata;* mass plot. Some of the points represent fifty beats but most of them, thirty. The slope is in accordance with the Arrhenius equation,

$$ln\ \frac{K_1}{K_0} = \frac{\mu}{2}\left(\frac{1}{T_0} - \frac{1}{T_1}\right)$$

For intermediate temperatures, $\mu = 11,200$.

It is hoped that the presentation of all the data will avoid the criticism that might be levelled at selected cases. These, in view of certain difficulties reported by Rywosch (1905), would not be altogether adequate. Indeed this author considered the heart of *Pterotrachea* essentially lawless because at any given temperature the rate may be quite different in different individuals. Rywosch also notes that

sudden changes such as plunging an animal from 15° to 40.5° and returning it after 1 minute to 16.5° may stop the heart at once or dissociate auricular from ventricular rhythm. Such drastic methods of course produce results quite different from moderate physical violence or small but abrupt changes in temperature. Indeed, these alone are enough to induce some of the effects demonstrated by Rywosch; they may even initiate disharmonies of rhythm that become noticeable in the rate, or bring the heart to a standstill, not immediately, but at some quite different and totally innocent point on the temperature

TABLE I.

Pterotrachea Heart.

Animal.	μ (intermediate).
1	11,200
2	11,900 (twice).
3	11,100
4	11,000
5	11,000
6	11,500
7	10,800
8	11,100
Average..........................	11,200

scale. Manipulative and experimental ineptitudes like these are perhaps useful in suggesting methods by which the heart might be controlled, but unfortunately they may also prove highly misleading.[1] From our general plot we should expect a reasonable uniformity among the increments deduced for separate individuals. This, as the list in Table I indicates, is true.

In selecting cases for individual presentation, it seemed best to choose the two that are farthest away from the average and the one that happens to exhibit the increment of 11,200. The data for these individuals are plotted in Fig. 2, and illustrate the precision with which

[1] Rywosch (*loc. cit.*, p. 361) writes: "Es lässt sich keine allgemeine Formel aufstellen in welchem Verhältniss etwa die Zunahme der Zahl der Pulse zur Erhöhung der Temperatur steht; ganz allgemein scheint es nur zu sein dass bei höheren Temperaturen auf eine Wärmegrad mehr Pulse kommen als bei niedrigeren."

the heart accelerates at intermediate temperatures. They also show, though not to greatest advantage, the terminal changes at extreme temperatures.
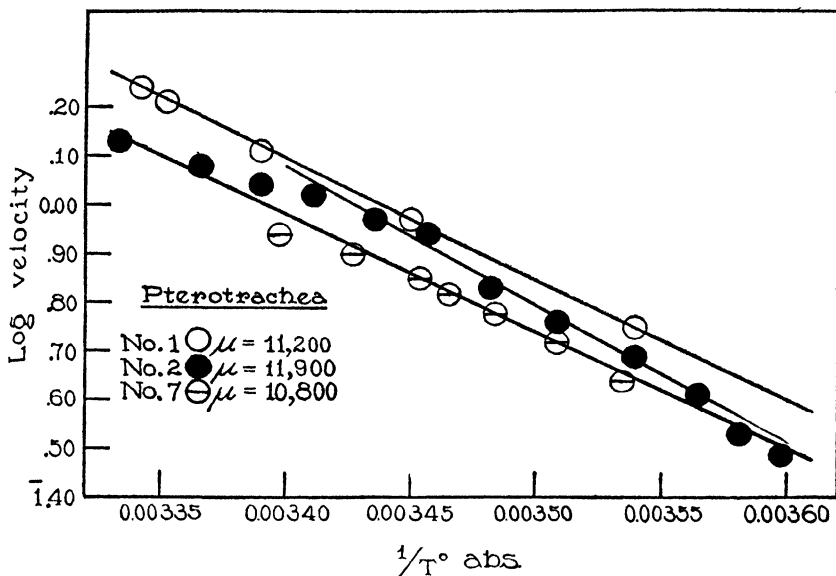

FIG. 2. *Pterotrachea;* heart rates of three animals, represented by different symbols. Of these individuals two give extreme values and one, 11,200. The increments are between 7,000 and 8,000 at high temperatures; and at low, in the neighborhood of 22,000. See Animals 1, 2, and 7, Tables I and III.

III.

Observations on the heart of *Tiedemannia* were made in exactly the same manner except that the aquaria, like the animals, were smaller. In the largest individuals moderate rhythmical movement of the "wings" facilitates the distribution of heat and does not obscure the view of the heart; in the smaller animals, however, it is often neces- sary to wait for periods of complete quiescence. In a general pres- entation of the data in a single plot, *Tiedemannia* in the middle ranges of these experiments exhibits an increment of 16,200.[2]

[2] Four out of eleven individuals exhibit an increment of 14,500±. The ex- planation cannot be found in manipulative irregularities committed in the course of experimentation. The points on which this increment is based could hardly demonstrate its reality with greater clearness. The matter will be discussed later.

These curves are in every way comparable with the results for *Pterotrachea.*
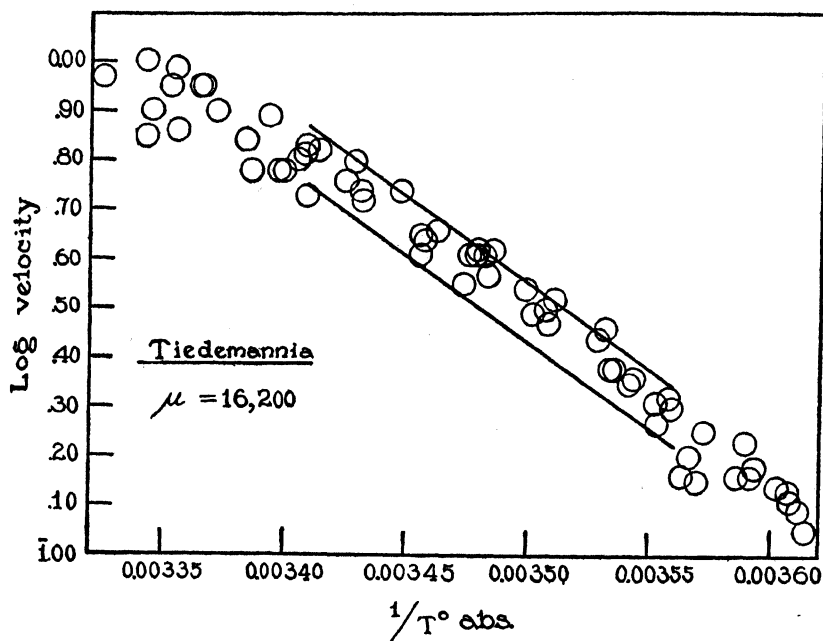

FIG. 3. *Tiedemannia neapolitana;* mass plot.   At intermediate temperatures, $\mu = 16,200$.

TABLE II.

Tiedemannia Heart.

Animal.	μ (intermediate).
1	16,000
2	16,300
3	16,200
5	16,100
6	16,500
9	16,100
10	16,100
Average.............................	16,200

IV.

Until further studies on the upper and lower limits of the temperature field are available, the results for these regions must be considered as essentially an orientation. Necessarily, observation in these distal ranges is greatly restricted. At both high and low temperatures certain individuals show no change of increment; whereas
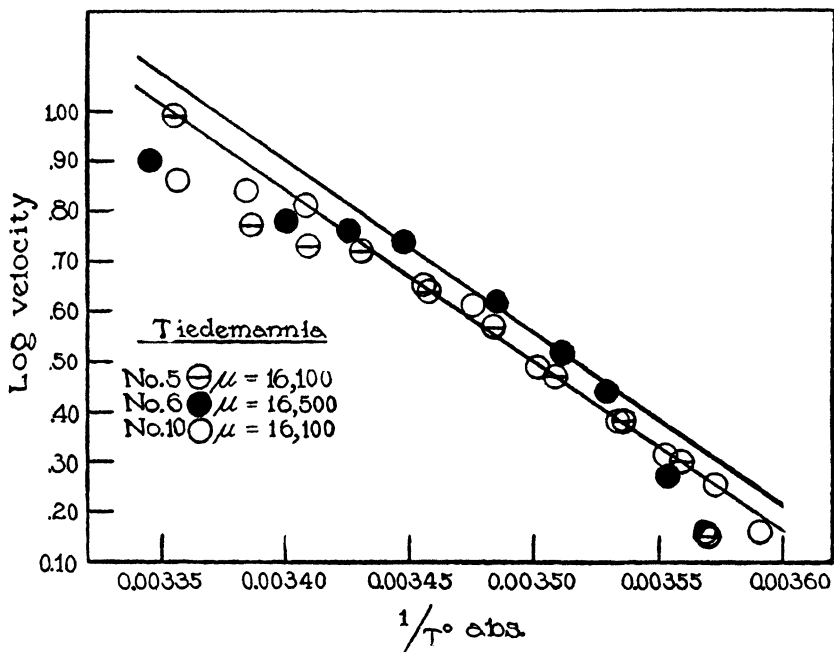
FIG. 4. *Tiedemannia;* heart rates of three individuals, see Animals 5, 6, and 10 Tables II and III. No. 5 illustrates the return to intermediate increment after an intermission at high temperatures.

in others, even with the exercise of great care, the heart beat becomes irregular, intermittent, or stops altogether. Near the upper limits, an intermission may be followed, significantly, by great and sudden variability. It is very difficult at times to be certain what the resulting points imply. Again, the onset of a shift in μ values appears to be influenced by the rate at which the temperature has been changed. Excluding the instances in which μ is either incalculable or unchanged,

it becomes relatively easy to rate the terminal increments as to their general order of magnitude with fair precision. As indicated in Table III, the values for μ at the extremes are sufficiently constant and different to suggest for *Pterotrachea* and *Tiedemannia* hearts the existence of apparently not less than two distinct terminal controls.

TABLE III.

Terminal Values for μ.

Pterotrachea No.	Temperatures.	
	High.	Low.
2	7,200	20,600
2	6,900	28,000
3	8,300	22,500
4	7,800	20,500
5	7,500	21,800
6	—	17,400
7	8,400	—
8	4,900	—
Average.................	7,300	22,000
Tiedemannia[*] No.		
1	7,000	—
3	9,200	20,100
4	5,900	21,700
5	7,000	—
6	7,200	27,800
7	8,900	20,900
8	7,400	26,400
9	7,200	—
10	5,900	—
Average...............	7,400	23,000

[*] This table includes the terminal data for the individuals with intermediate increments of $14,500\pm$. These it will be observed are indistinguishable from those where the intermediate $\mu = 16,200\pm$.

V.

There can be no doubt that the controlling mechanisms at high and low temperatures are different. The harmony between the comparable averages for *Pterotrachea* and *Tiedemannia* is astonishing

and very possibly not an accident. Yet within each list of high and low values, there are striking discrepancies. In the records, certain of these "increments" far above the average, and others as far below are attested by observations that can be interpreted, at present, in no other way. The precise meaning of these extreme variates and of certain intermediate irregularities remains problematical. Despite the remarkable agreements noted, certain temperatures, conceivably, may uncover in the heart rate one or more of a variety of processes which at other temperatures remain concealed. If this is a fact, then in certain states of equilibrium any one of these recessive reactions might be "exhumed" and fall momentarily into the position of control.

However, if we consider only the most frequent and closely concordant values, it seems safe to say that high temperatures disclose at least one controlling reaction whose characteristic lies between 7,000 and 8,000, while low temperatures uncover a second with its increment in the neighborhood of 22,000.

Are these or perhaps any of the other terminal values normal? From a purely physical standpoint this question is equivalent to asking whether a heart forced to disclose either both, or any, of the terminal increments, can return to the conditions that yield 11,200 or 16,200. Very likely if the animals were kept long enough at the extremes, the mechanism underlying the heart beat could be permanently altered. In these experiments the exposures were not sufficient to bring about such results. In every case a set of preliminary readings was taken at room temperature soon after the material had been brought into the laboratory. The animals were then cooled to the lower limit and when this was reached, usually after an hour, the first definitive observations were made. From this point on the temperature was increased step by step at regular intervals up to the maximum. In the intermediate zone, therefore, the performance of each individual occurred only after exposure to the lower thermal limit. The first reading at room temperature always coincides with the corresponding one made later on as part of the ascending series.

For the low values found at the upper limits, there are three checks. First, their general order of magnitude seems to rule out destructive effects such as irreversible coagulations or precipitations of proteins;

second, when the heart stops and after an intermission returns to rhythmical contraction with chambers properly synchronized, the results abruptly approach, or actually fall on the line characteristic for intermediate temperatures. This fact which at times makes the recognition of terminal increments difficult or impossible, is not without its compensations. Other possibilities granted, it suggests that during intermissions some essential material, exhausted, depleted, or "inactivated" by the great increase in heart rate, is restored to its original state or amount. Finally, in the case of *Pterotrachea* there is a special test. Individual 2 was run through the entire series of temperatures on 2 successive days. On January 26 in the range from 4–27°, the successive μ values were 20,600, 11,900, and 7,200; on January 27, from 4.6–26.8°, this animal gave respectively, 28,000, 11,900, and 6,900. Judging by their sense and absolute values and in view of the other considerations, these increments suggest no radical alterations in the underlying mechanism.

<div align="center">VI.</div>

Within present limits of accuracy the intermediate increments of *Pterotrachea* and *Anodonta* on the one hand, and on the other of *Tiedemannia*, and *Limax* (Crozier and Stier), are identical. In sense and as averages the corresponding terminal characteristics for *Pterotrachea* and *Tiedemannia* are also the same. But homogeneity does not end with these indications. The fact that certain species exhibit increments of 16,000 ± for rhythmic movements under circumstances which elicit 11,000 ± from others, loses the qualities of an obstacle when we recall that in *Melanoplus* Crozier and Stier (1924–25, *b*) have experimentally changed the increment for abdominal rhythm from 16,500 to 11,300. For *Limax* the same authors (1924–25, *a*) find intermediate increments of 7,900 in December and 16,300 in March; while from Lang's results on *Helix*, they have deduced for the same temperatures μ values of 7,900 from data gathered in June, 11,300 from those of January, whereas February and March yielded 16,000. In all these instances the reaction in control at specific temperatures evidently differs as circumstances differ, yet the values at whatever temperature found, are always the same ones. What are the implications? Certainly in view of the taxonomic differ-

ences distinctive of the molluscs under consideration it would be hazardous to assume more than one catenary series of controlling reactions. Within the limits of viability, even this series, should further work lengthen it considerably, must be thought of as including at all times at least as many processes as there are trustworthy increments. Under certain conditions, most of which await precise definition, a particular region of the thermal scale may expose one reaction while under other circumstances the same temperatures may unearth quite another. Indeed, with the sole exception of artefacts or novelties introduced into the organism from without, a thermal field, biologically defined, can expose only normal constituents of the catenary system of control.

The logical consequences of this reasoning may appear ominous; and yet confusion and absurdity can be avoided even if thermal increments were to multiply equally for every biological process. After all, the total number of increments possible is probably restricted (Rice, 1923; Glaser, 1923; 1924–25, b). It is not difficult to imagine that circumstances might arise under which control could pass to chemical reactions which are not ordinarily in control. Our theory, at least, must be able to face the organism which discloses for all its measurable acts the same inventory of increments with differences only in serial order. In this somewhat academic event, analysis by means of temperature characteristics would be exhaustive because the series of catenary controls would contain as many items as there are types of reaction in the actual chemical system. If we could know where and how many times each type occurs, our diagnostic series and the actual system could be superimposed, point for point.[3]

[3] The addition of every authentic increment is a step in the direction of the theoretical limit. For this reason the occurrence of two intermediate μ values in *Tiedemannia* seems important despite the absence of a convincing explanation. It is possible to assume differences in prelaboratory history, but whatever these may have been, the terminal increments remain unchanged. The lower value is not necessarily irreconcilable. Rare as it is, increments of this order associated with $11,000\pm$ and $16,000\pm$ have been reported in connection with respiratory processes and those that might be limited by the rates of gaseous exchange. Arrhenius (*loc. cit.*, p. 55) lists as a mean value for cell division in certain eggs, 14,100 and attaches to "respiration in plants," 14,800. According to Crozier (1924–25, b), Krogh's data on respiration in dogs under curare yield $\mu = 13,780$.

VII.

The low increments characteristic of high temperatures are well supported in the literature. Somewhere in the catenary chain underlying respiratory phenomena in general and phenomena controlled by respiration, is a reaction whose increment is between 7,000 and 8,000. In certain instances, such as translation in *Paramecium* (Glaser, 1924–25, *a*) and the heart rates of *Pterotrachea* and *Tiedemannia*, it requires a fairly high temperature to expose this reaction whereas in *Melanoplus*, as Crozier and Stier (1924–25, *b*) show, it may control in normal or decapitated individuals over the entire range. To these writers (*loc. cit.*) also, we owe an interesting suggestion. Between 15° and 38° Bodine's measurements on CO_2 production in *Melanoplus* yield $\mu = 7,710 \pm 700$, whereas the data of Batelli and Stern (Crozier and Stier), perhaps doubtful on account of the high temperatures employed (between 30° and 40°), yield an increment of 8,000 ±. Crozier and Stier plainly imply a relation between this value and the excretion of CO_2.

For the increment of 22,000 ± at low temperatures, a provisional assignment is perhaps also possible. It is found frequently associated with 11,000 ± and 16,000 ± (Crozier, 1924–25, *b*). Under laboratory conditions the blood of *Panulirus* undergoes a rapid loss of

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With the exception of cleavage, all these processes under different circumstances have also given $\mu = 16,000\pm$ or $11,000\pm$, or both. It is not impossible then that increments of $14,000\pm$ are normally recessive in respiratory phenomena but for one reason or another, not apt to emerge.

The association of this value with curare is suggestive. *Tiedemannia*, as observed, moves its "wings" rhythmically. If the individual happens to be a small one it is often necessary to await those recurrent periods when the individual is completely quiescent. Just prior to such an interval the movements become slower and the excursions more prolonged—symptoms found during the rhythmical phase of reflexes approaching fatigue (Bayliss, 1924; p. 501). If we suppose that *Tiedemannia* comes to rest for the same or comparable reasons, an explanation for the value $14,500\pm$ might be sought and perhaps found in the element common to the effects of curare and of certain fatigue substances, curare eliminating the final motor neurone by affecting a "receptive" material in the muscle and "fatigue" deleting the same neurone by blockade at the proximal synapse (*cf.* Bayliss, *loc. cit.*, p. 399 *et seq.*). Until the situation is clarified, however, we must withhold from the increment of $14,500\pm$ the consideration given to the others.

glucose (Morgulis; *cf.* Crozier, 1924–25, *b*). In starving crayfish, Brunow's figures on the utilization of O_2 and the production of CO_2 yield μ = 22,000 (Crozier, *loc. cit.*). It is easy to imagine that low temperatures might reduce, possibly reduce differentially, either the solubilities or the rates upon which a normal supply of glucose depends. If this is true, such temperatures would bring about changes in equilibrium closely comparable and perhaps identical with those characteristic of inanition. The temptation to attach 22,000 to hydrolytic reactions in which a substrate is prepared for oxidation is recognized by Crozier (*loc. cit.*).

<div align="center">VIII.</div>

We can now attempt to picture the controlling mechanism for heart rate in molluscs, provisionally, as a catenary series of at least four reactions. Of these, the one with the smallest increment controls at high temperatures and the one with the largest value at low. Between these extremes control may fall to either of the remaining two, and these, as in *Melanoplus*, should be capable of being artificially substituted, one for the other.

Although we are not dealing exclusively with a muscle, it is perhaps not unreasonable to consider glycogen as a source of energy. Assuming an adequate original supply, O, a mechanism modelled on Meyerhof's (1924) conception of carbohydrate metabolism appears capable of accounting for the observed velocity controls. We can attach the increment of 22,000 $\pm$, directly or *via* some catalyst, to a mobilization hydrolysis, $O \rightarrow A$. In a similar manner 11,000 $\pm$ may be assigned to an "oxidation," $A \rightarrow B$, very likely catalyzed by OH′; while 16,000 $\pm$ may be taken to characterize a reaction $B \rightarrow E$ which liberates the energy and is catalyzed otherwise. The increment 7,000 $\pm$ would then fall to the resynthesis $E \rightarrow O$ (lactic acid $\rightarrow$ glycogen?), and on occasion also to any other pertinent reaction involving the production of CO_2 and important in maintaining the original source of supply.

While the interpretations on which this particular scheme is based should involve no irrevocable commitments, it has, nevertheless, certain advantages. For one thing, its form projects into the substrate the cyclical nature of rhythm, and, resting on the apparently

solid foundation laid by Meyerhof (*loc. cit.*) brings under one rubric phenomena superficially diverse. As a formulation we are obviously dealing merely with the harmonious enlargement of an idea suggested as applicable to linear translation in *Paramecium* (Glaser, 1924–25, *a*), where the thermal field so far has exposed only two values: 16,000 from 6–15° and 8,000 for the higher ranges. The interpretation of these values remains unchanged. As Crozier and his collaborators (1924–25) have shown, increments of 11,000 $\pm$ and 16,000 $\pm$ are characteristic of processes involving O_2 consumption and the produc-

Oxidation catalyzed by OH' $\mu = 11,200\pm$

Oxidation catalyzed by Fe or ? $\mu = 16,200\pm$

Mobilization hydrolysis? $\mu = 22,000\pm$

Resynthesis limited by CO_2 excretion? $\mu = 7,000\pm$ $\mu = 8,000\pm$
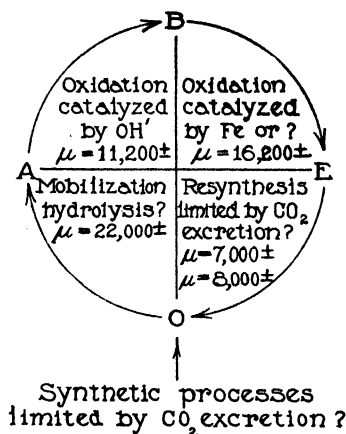
Synthetic processes limited by CO_2 excretion ?

FIG. 5.

tion of CO_2. Of these values, the former is believed to reveal the activity of OH' (*cf.* Rice, 1923), although the reaction in which this intervention occurs may itself issue in oxidation (Oppenheimer, 1925, page 225); the second, 16,000 $\pm$ is perhaps definitely to be associated with a catalyzed oxidation. The assignment of 22,000 $\pm$ to a mobilization hydrolysis is admittedly the most speculative of all. On the other hand, for the lowest increment we can appeal to at least one other synthetic process. In the data of Miss Leitch (Crozier, 1924–25, *b*) μ below 15° is 8,170 for growth in the radicle of *Pisum*. If in the synthesis $E \rightarrow O$ the excretion of CO_2 should be a limiting factor, it is interesting to recall (Meyerhof) that the reaction which transforms lactic acid into glycogen also sets free six molecules of CO_2. Finally, the place assigned to the lowest increment makes it

conceptually easy to link the immediate cycle underlying the heart rate with the rest of the organism because the synthesis of O is not restricted to movements from E, but in the model may result, as it does in nature, from reactions spatially distinct yet with the same control.

SUMMARY.

1. For the heart rate in *Pterotrachea coronata*, intermediate temperatures disclose a thermal increment of 11,200 $\pm$. This value is identical with the one reported by Crozier and Stier for the lamellibranch, *Anodonta*. In the pteropod, *Tiedemannia neapolitana* the same temperatures typically reveal in the heart rate a μ value of 16,200 $\pm$. This agrees quantitatively with 16,300 found by Crozier and Stier for the heart of the slug, *Limax maximus*.

2. At high temperatures the average value of μ for *Pterotrachea* is 7,300: for *Tiedemannia*, 7,400. The corresponding averages at the lower limits are 22,000 and 23,000.

3. The great variability found near the edges of the temperature field are explicable in two ways. During intermissions characteristic of high temperatures and occurring also at low, we can assume a restorative process; while at both the upper and lower limits we may, in addition, find that reactions assume control which under ordinary circumstances never do so. Special evidence indicates that the highest temperatures employed, 27°C., and the lowest, 4°C., caused no irreversible changes in mechanism.

4. The theoretical analysis of the experimental facts makes use of Meyerhof's conception of carbohydrate metabolism and projects the cyclical nature of rhythm into the substrate of control. Assuming as a source of energy an original supply of material O, the value of 22,000 $\pm$ is assigned provisionally to a mobilization hydrolysis while 11,200 $\pm$ and 16,000 $\pm$ are attached to oxidative reactions influenced respectively by OH' and possibly Fe, or some other catalyst. The lowest value, 7,300 $\pm$ is assumed to indicate a synthetic process (lactic acid → glycogen?), possibly limited by CO_2 excretion. In the present state of our knowledge, this distribution and interpretation seems to account reasonably for the experimental facts, but until we know more about the neurogenic controls, is entitled to rank only as an hypothesis.

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TIME RELATIONS OF GROWTH.

II. The Equivalence of Age in Mammals Estimated on the Basis of Their Growth Constants.*

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I.

INTRODUCTION.

In the preceding communication of this series,[1] it was explained that the smoothed curves of growth in weight of animals are usually sigmoid, yet they have the point of inflection not in the center of the curve, but where slightly over one-third of the mature body weight is reached; and that after the point of inflection the course of growth in weight may be represented by the exponential equation

$$W = A - Be^{-kt} \tag{1}$$

In this equation, W represents the weight of the animal at the age t, A represents the weight of the animal at full maturity, e is the natural base of logarithms, B is a constant the significance of which was explained in detail in the preceding communication, and k represents the fractional decline in growth. It is, of course, evident from equation (1) that for the period of growth for which the equation holds true, the velocity of growth (that is the gain in weight per unit time) declines in a geometrical progression with age, and that k represents the fractional decline in the velocity of growth.

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[1] Brody, S., *J. Gen. Physiol.*, 1925–26, viii, 233.

Equation (1) is a compact description of the time relations of growth representing a period of growth during which nearly two-thirds of the mature body weight is gained. It contains no empirical constants and, as was suggested in the preceding communication, it may have a rational basis in the mechanism underlying the decline in growth. This equation may, therefore, be tentatively considered as a law of growth during the period of declining growth velocity.
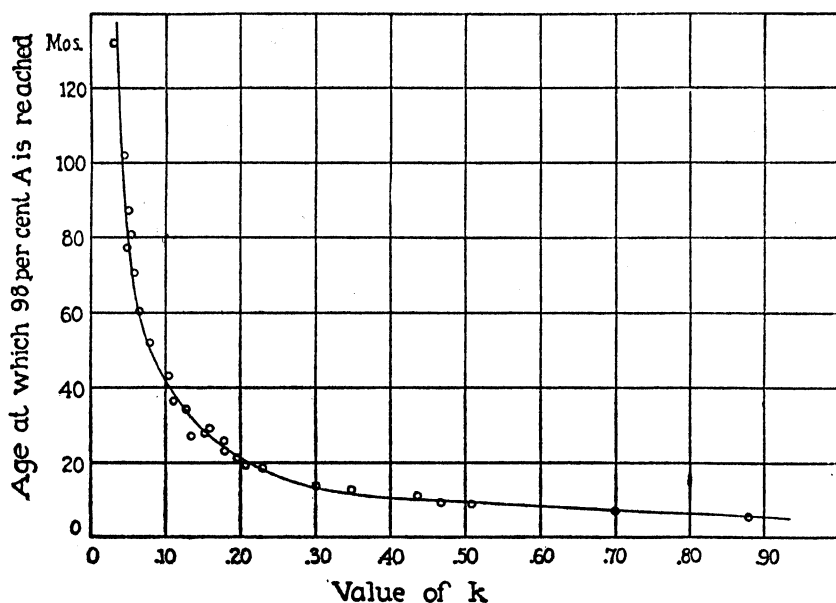

FIG. 1. The relation between the numerical values of the velocity constants of growth, k, and the time required to reach 98 per cent of the mature value, A, in different animals.

II.

Equivalence of Age.

The constant k in equation (1) has a very definite meaning; it represents, as already explained, the fractional decline in the velocity of growth with age. The greater the fractional decline, k, the more rapidly will the limiting or mature value A be approached. Indeed, the rapidity of approach to the mature weight, A, is directly proportional to the numerical value of k, as shown in Fig. 1, and the relative
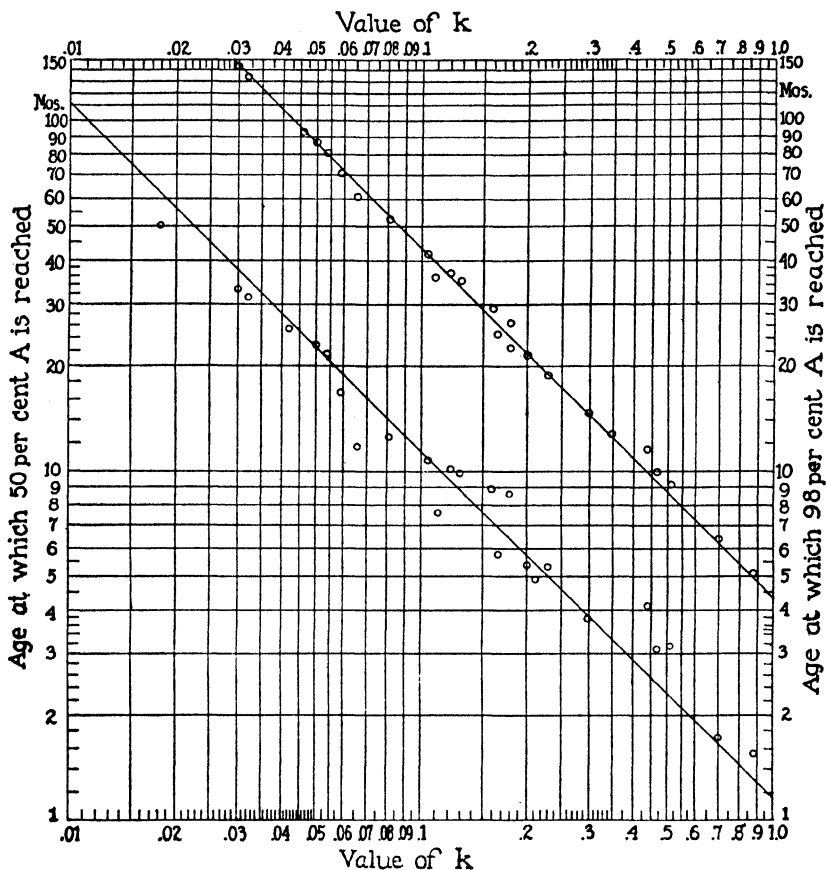
FIG. 1, *a*. The relation between the numerical value of the growth constant, *k*, in different animals and the time required to reach 98 per cent of the mature weight (upper line) and 50 per cent of the mature weight (lower line) plotted on logarithmic coordinate paper. The linear distribution of the data on this paper indicates that the time, *t*, required to reach a given fraction of the mature weight is inversely proportional to *k*. That is

$$t = \frac{c}{k}$$

c is a constant. *c* has a value of 4.35 when *t* represents the time required to reach 98 per cent *A*; *c* = 1.117 when *t* represents the time required to reach 50 per cent *A*.

The data on the 50 per cent line are less evenly distributed than on the 98 per cent line due to the fact that at the lower levels the period of growth preceding the point of inflection with which equation (1) is not concerned becomes a factor of relatively increasing importance.

duration of the periods of growth of two animals is, therefore, inversely proportional to the numerical values of their k's. These facts give us a basis for computing the equivalence of growth age in different animals.

TABLE I.

Equivalence of Age Following the Point of Inflection; also Equivalence of Weight.

	Age equivalence.			Weight equivalence.		
	Referred to 1 unit in the Jersey cow as a standard.	Referred to 1 unit in the male guinea pig as a standard.	Referred to 1 unit in the unmated female white rat as a standard.	Referred to 1 unit in the Jersey cow as a standard.	A (Mature weight.)	k
					kg.	
Jersey cow (Eckles)................	1.000	4.26	11.9	1.000	420	.054
Ayrshire cow (Eckles)..............	1.080	4.60	12.9	1.100	459	.050
Holstein cow (Eckles)..............	1.174	5.00	14.1	1.300	550	.046
Duroc-Jersey swine, females (F. B. Mumford)......................	.870	3.70	10.4	.480	200	.062
Suffolk sheep, females (Murray)......	.292	1.24	3.48	.190	80	.185
Shropshire-Merino sheep, females (Murray).......................	.287	1.22	3.43	.119	50	.188
Guinea pig, males (Wright)..........	.235	1.00	2.80	.002	.825	.230
White rat, unmated females (Donaldson, Dunn, and Watson)..........	.084	.357	1.00	.00048	.203	.644
White rat, males (Donaldson, Dunn, and Watson)....................	.135	.575	1.61	.00067	.280	.400
White rat, males (Greenman and Duhring)......................	.154	.657	1.84	.00083	.350	.350
Norway rat, males (King).............	.443	1.88	5.28	.00092	.385	.115
White mouse, males (Robertson).......	.087	.371	1.04	.000065	.0275	.620
White mouse, females (Robertson)......	.066	.280	.785	.000056	.0235	.820

Since the preparation of this table and Figs. 13 and 14, the value of k for the Norway rat was changed from .115 to .122.

Thus, from Table I of the preceding paper, the numerical value of k of the Jersey cow is .054; of the female white rat it is .644. Therefore, 1 month in the rat is equivalent (during the phase of growth following the point of inflection) to $1 \times \dfrac{.644}{.054}$ or 11.9 months in the

cow; or 1 month in the cow is equivalent to $1 \times \dfrac{.054}{.664}$ or .08 months in the cow and 1 month in the white rat are given in Table I.
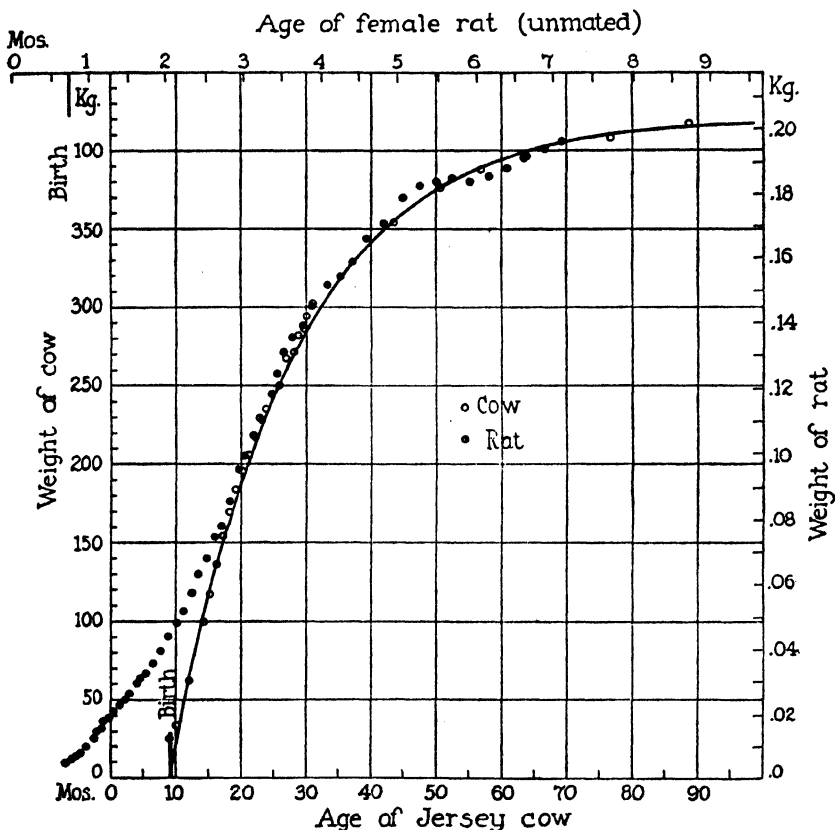

FIG. 2. Growth-equivalence of Jersey cow and unmated female white rat. After the point of inflection, 1 month in the rat is equivalent to 11.9 months in the cow and 1 gm. of weight in the rat is equivalent to 2.068 kilos in the cow.

The period of growth preceding the point of inflection is relatively longer in the rat than in the cow.

Growth in weight-equivalence may be similarly computed by comparing the numerical values of the mature weights A. The value of A for the Jersey cow is 420 kilos; of the female rat it is .203 kilos.

Therefore, 1 gm. in the rat corresponds to 2.07 kilos in the cow. Such equivalent values on the basis of the Jersey cow are given in Table I.
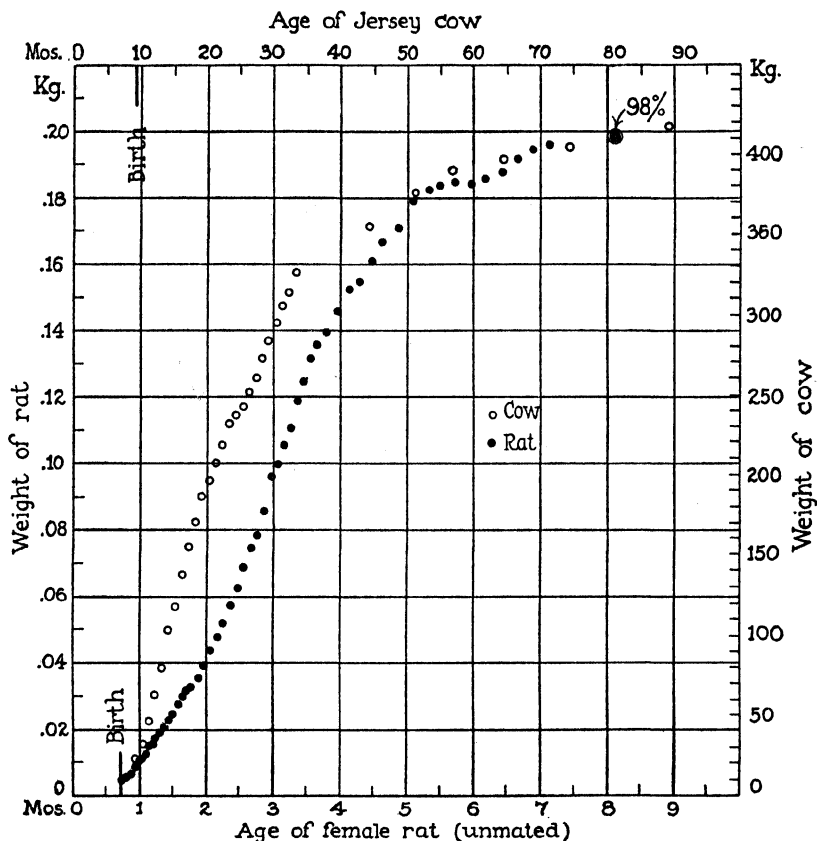

FIG. 2, *a*. Growth-equivalence of cow and unmated female white rat obtained on the assumption that the age when 98 per cent of the mature weight is reached is equivalent in the two animal forms. Compare with Fig. 2.

III.

Constructing Equivalence Charts with the Aid of the Numerical Values of k and A.

Having elaborated a method for determining equivalence of growth age, we have proceeded to compare growth curves of animals in order to learn something of the distinctive peculiarities of growth curves

of different animal forms, and incidentally to test our method of determining equivalence.

The results of these comparisons are shown in Figs. 2 to 13. In most of the graphs, the Jersey cow is taken for the standard of comparison. The observed values are represented by circles, the values computed from equation (1) are represented by smooth curves. The observed values of the animals under comparison agree satisfactorily except at the very early stages of growth with which, as pointed out, equation (1) is not concerned. The agreements between the observed values and the values computed from equation (1) are also satisfactory for the phase of growth following the points of inflection which is the only phase of growth under consideration.

The equivalence charts are self explanatory and they make more interesting reading than any comments we may offer in their behalf. It only remains to illustrate the method of preparing an equivalence chart.

We may take, for purposes of illustration, the steps involved in preparing an equivalence chart for the Jersey cow and the female white rat (Fig. 2). We have found, as already explained in the preceding section, that

$$1 \text{ month in the cow} = \frac{.054}{.644} \text{ or } .084 \text{ months in the rat}$$

$$\text{or}$$

$$1 \text{ month in the rat} = 11.91 \text{ months in the cow}$$

Now this statement does not imply that a 1 month old rat is physiologically as old as an 11.9 months old cow; for, as pointed out, this equivalence was obtained on the basis of the numerical values of the k's in equation (1) for the cow and rat, and equation (1) represents only the phase of growth following the point of inflection in the smoothed growth curve. The above relation, which was found to hold true for the phase of growth following the point of inflection, may or may not hold true for the phase of growth preceding the point of inflection. As a matter of fact, this relation does not hold true for the rat and the cow for the phase of growth preceding inflection (cf. Figs. 2 and 2, a). For the cow and guinea pig, on the other hand, the relation between their k's also applies for the phase of growth preceding inflection (cf. Figs. 3 and 3, a).

All this is only equivalent to saying that conception cannot be
taken as a point of reference in preparing equivalence charts which
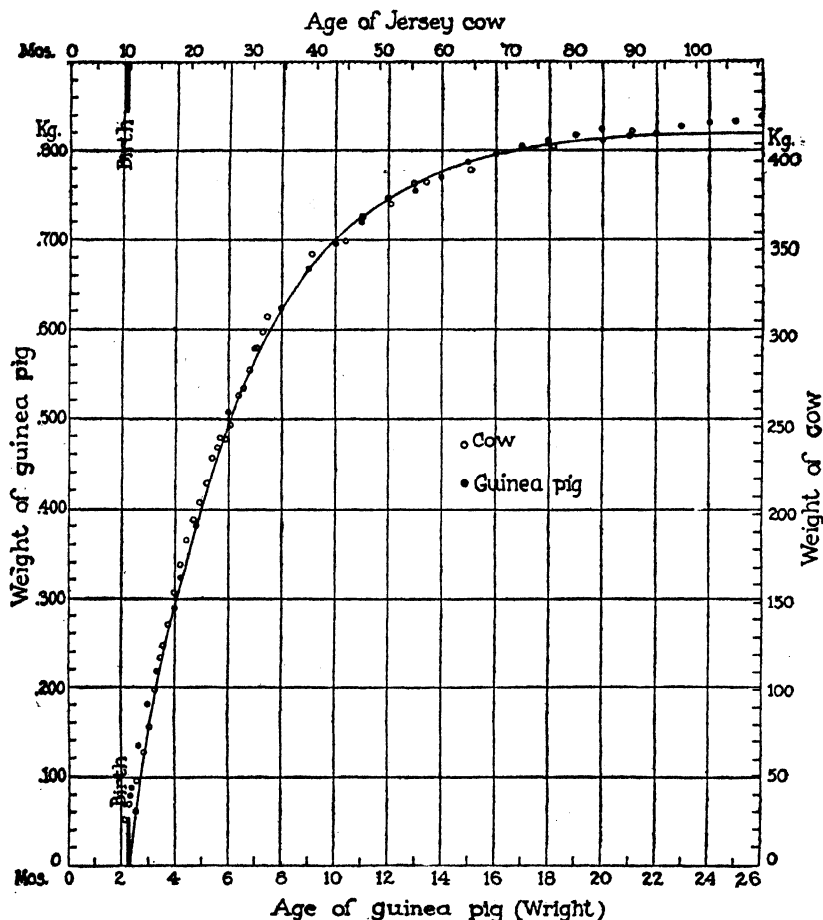
FIG. 3. Growth-equivalence of Jersey cow and male guinea pig. 1 month in
the guinea pig is equivalent to 4.26 months in the cow and 1 gm. in the guinea pig
is equivalent to 509.1 gm. in the cow.

Unlike the case of the rat, growth in the guinea pig appears to follow the same
course as growth in the cow from conception.

should represent equivalence of growth in weight following the point
of inflection.   Instead of taking conception we take for the points of

reference the ages when the curve of equation (1) cuts the age axis. We termed this age t^*, and its significance is illustrated in detail in Figs. 2 and 3 of the preceding communication. It is only after the

FIG. 3, *a*. Growth-equivalence of cow and guinea pig obtained on the assumption that the age when 98 per cent of the mature weight is reached is equivalent in the two animal forms. Compare to Figs. 2, 2, *a* and 3.

age t^* that 1 month in the rat is equivalent to 11.9 months in the cow.

The numerical value of t^* may either. be read from the graph of Figs. 2 and 3 in the preceding communication, or it may be computed
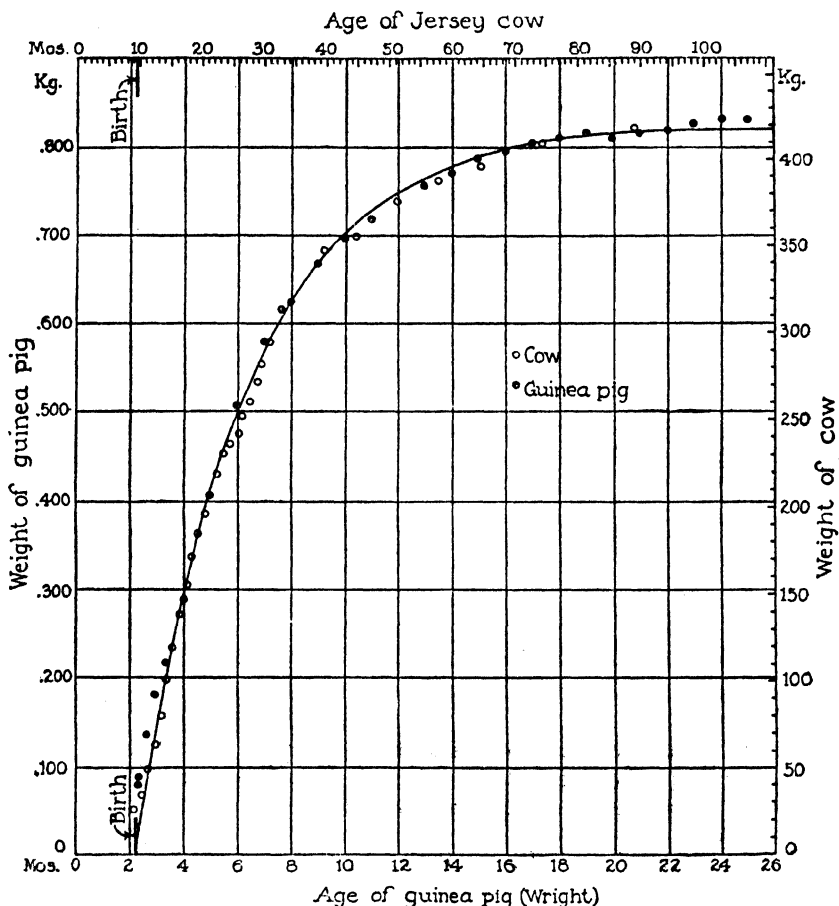
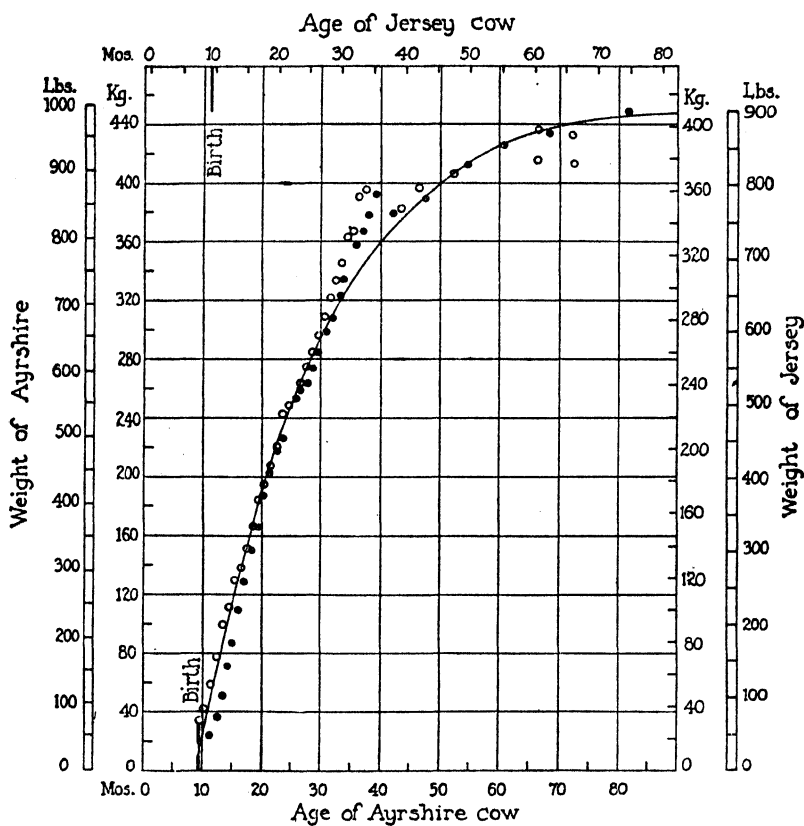FIG. 4. Growth-equivalence of Jersey cow and Ayrshire cow. 1 month in the Jersey cow is equivalent to 1.08 months in the Ayrshire cow; and 1 kilo in the Jersey cow is equivalent to 1.1 kilos in the Ayrshire cow.

as follows: when $t = t^*$, $W = 0$ (by hypothesis), and equation (1) becomes

$$0 = A - Be^{-kt^*}$$

$$A = Be^{-kt^*}$$

$$\log_e A = \log_e B - kt^*$$

$$t^* = \frac{\log_e B - \log_e A}{k}$$

The work is now laid out as follows:

	A (Mature weight.)	k	t^*	
	kg.		mos.	Age factor = $\dfrac{.644}{.054}$ = 11.91
Unmated female white rat.......	.203	.644	2.03	
Jersey cow....................	420.	.054	8.9	

The values of t^* in the cow and rat, namely 2.03 and 8.9 months, constitute by hypothesis one pair of corresponding points. Another pair of corresponding values may be found as follows: Let us take 8 months from conception in the rat or $8.00 - 2.03 = 5.97$ months from t^* in the rat as another point of reference. 5.97 months in the rat is equivalent to $5.97 \times 11.91 = 71.1$ months in the cow from t^* or $71.1 + 8.9 = 80.0$ months in the cow from conception. 8 months in the rat and 80 months in the cow (counted from conception) thus constitute the second pair of corresponding points in the ages of the cow and rat.

For weight equivalence, zero in the rat corresponds to zero in the cow and .203 kilos in the rat corresponds to 420 kilos in the cow. It only remains to uniformly graduate the axes between the points of reference, and to extrapolate the graduations if desired.

IV.

Constructing Equivalence Charts on the Basis of Conception and the Age When 98 Per Cent of the Mature Weight Is Reached.

We have also attempted to obtain equivalence of age values by taking conception and some convenient fraction of the mature weight A as points of reference. This method is similar in principle to the method employed by Pearl and coworkers[2] for comparing mortality curves of man, *Drosophila*, and *Proales decipiens*. This method of determining equivalence would be entirely satisfactory if the ratios between the segments of the growth preceding the point of inflection to the segments following it were the same in the animals under

[2] Pearl, R., *Am. Naturalist*, 1922, lvi, 398; *Metron*, 1923, ii, 1. Pearl, R., and Doering, C. R., *Science*, 1923, lvii, 209. Pearl, R., and Parker, S. L., *Am. Naturalist*, 1924, lviii, 71.

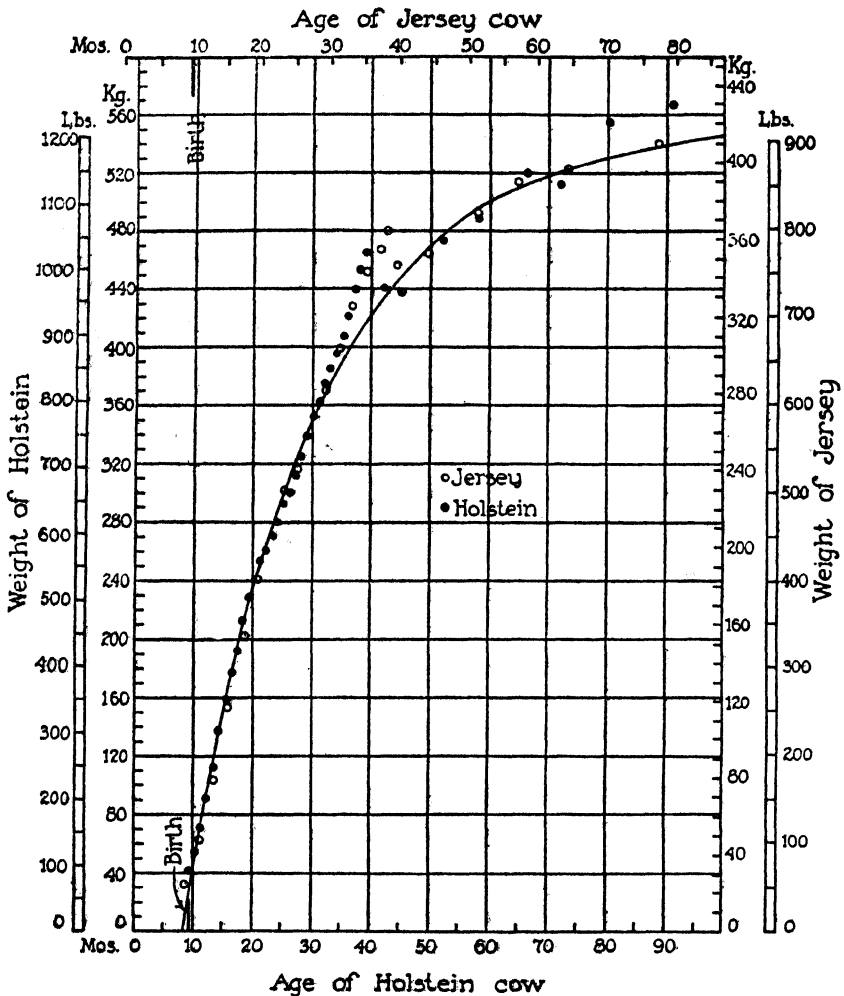FIG. 5. Growth-equivalence of Jersey cow and Holstein cow. 1 month in the Jersey cow is equivalent to 1.174 months in the Holstein cow and 1 kilo in the Jersey cow is equivalent to 1.3 kilos in the Holstein cow.

investigation. This is the case for the Jersey cow and male guinea pig. The agreement between the curves of these two animals is as good by the use of this method (Fig. 3, a) as it is by the method of

comparing the k's and t^*'s (Fig. 3). But the agreement between the cow and rat (Fig. 2, a) is very poor by this method throughout the whole curve while by the use of the preceding method (Fig. 2) it was good at least after the point of inflection. Both methods are useful
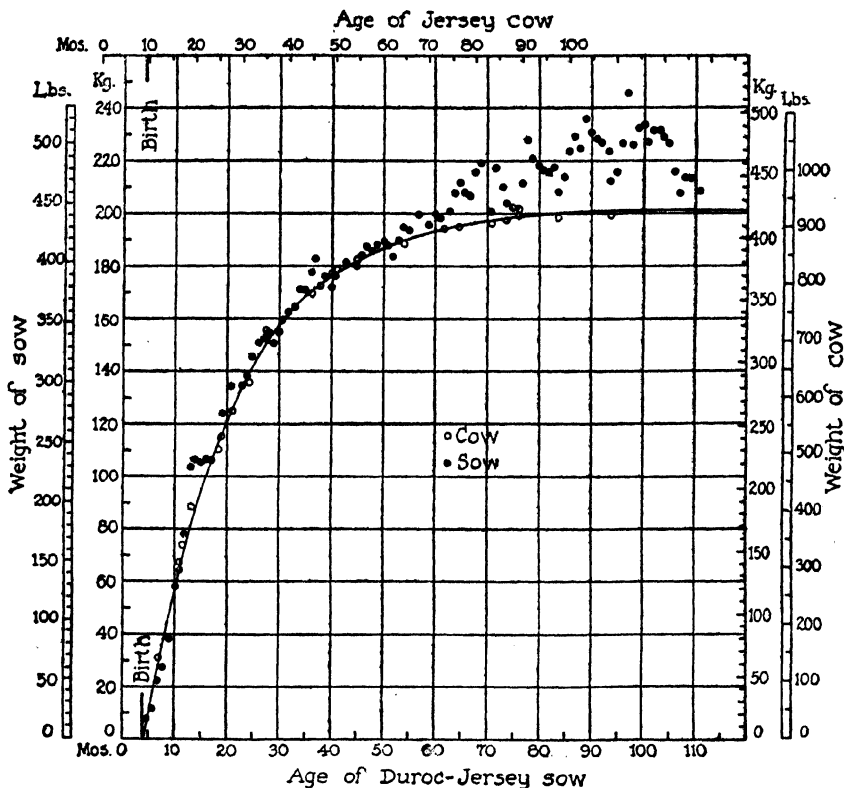
FIG. 6. Growth-equivalence of Jersey cow and Duroc-Jersey swine. 1 month in the sow is equivalent (after the point of inflection) to 1.15 months in the cow, and 1 kilo in the sow is equivalent to 2.1 kilos in the cow. The period before the point of inflection appears to be longer in the cow than in the sow.

depending on the viewpoint of the investigators; but, of course, neither method is entirely satisfactory if the several phases of growth do not occupy proportional parts of the growth curve in the animals under comparison.

TABLE II.

Equivalence of Growth Age.

Ages in months counted from conception when different fractions of the mature weight, A, are reached. The upper rows represent ages as interpolated from growth curves in which the observed values were connected by straight lines; the lower rows represent ages as computed from formula (3).

Percentage of mature weight	10	20	30	40	50	60	70	80	90	95	98	Mature weight. kg.
Holstein cow (Eckles)	10.5	13.5	16.0	19.5	23.8	28.7	34.0	43.0	59.0	73.4	93.2	550
	10.6	13.1	16.0	19.4	23.4	28.2	34.5	43.3	58.4			
Ayshire cow (Eckles)	10.8	13.4	16.0	19.4	23.0	27.4	32.8	41.4	55.5	69.0	87.3	460
	11.2	13.6	16.2	19.3	23.0	27.4	33.2	41.3	55.1			
Jersey cow (Eckles)	10.9	13.6	15.8	18.4	22.0	26.4	30.5	38.3	53.0	64.3	81.4	420
	10.9	13.1	15.5	18.4	21.8	25.9	31.2	38.7	51.6			
Duroc-Jersey sow (Mumford)	6.7	9.2	10.7	12.7	15.8	19.5	24.2	31.0	43.0	54.0	70.5	200
	5.9	7.9	10.1	12.7	15.7	19.5	24.3	31.0	42.4	53.9	67.3	
Suffolk ewe (Murray)	5.1	6.0	6.8	7.6	8.5	9.8	11.3	13.6	17.2	21.4	26.5	80
	5.3	6.0	6.7	7.6	8.6	9.8	11.4	13.7	17.5			
Guinea pig, male (Wright)	2.7	3.2	3.7	4.3	5.2	6.1	7.2	8.8	12.0	14.4	18.8	.825
	2.8	3.4	3.9	4.5	5.3	6.3	7.5	9.3	12.3	15.4	18.9	
White rat, unmated female (Donaldson et al.)	1.4	2.0	2.4	2.8	3.1	3.4	3.9	4.5	5.4	6.8	8.1	.203
	2.2	2.4	2.6	2.8	3.1	3.5	3.9	4.5	5.6	6.7		
White rat, male (Donaldson et al.)	1.6	2.4	2.8	3.1	3.6	4.1	4.9	5.7	7.9	7.4	11.7	.280
	2.1	2.4	2.8	3.1	3.6	4.1	4.9	5.9	7.6			
White rat, male (Greenman and Duhring)	1.6	2.3	2.8	3.3	4.0	4.4	5.0	6.3	8.4	10.1	13.1	.350
	2.0	2.4	2.8	3.2	3.4	4.4	5.2	6.3	8.4	10.0	12.9	
Norway rat, male (King)	1.9	2.8	3.9	5.7	7.0	9.1	11.8	15.7	20.3	38.0	34.9	.400
	1.8	2.8	4.0	5.3	7.0	8.9	11.4	14.9	20.9			
White mouse, female (Robertson)	.85	1.2	1.4	1.5	1.9	2.2	2.6	3.3	3.9	5.3	6.7	.0235
	.92	1.1	1.3	1.6	1.9	2.2	2.7	3.3	4.4	5.5	7.0	
White mouse, male (Robertson)	.95	1.1	1.3	1.5	1.8	2.0	2.3	2.7	3.9	4.8	5.5	.0275
	1.1	1.2	1.3	1.5	1.8	2.0	2.3	2.8	3.7	4.6	5.6	

V.

A Table of Equivalence of Age.

The equivalence charts are supplemented by a table of equivalent growth ages, or by a table giving the ages at which different fractions of the mature weight, A, are reached (Table II).
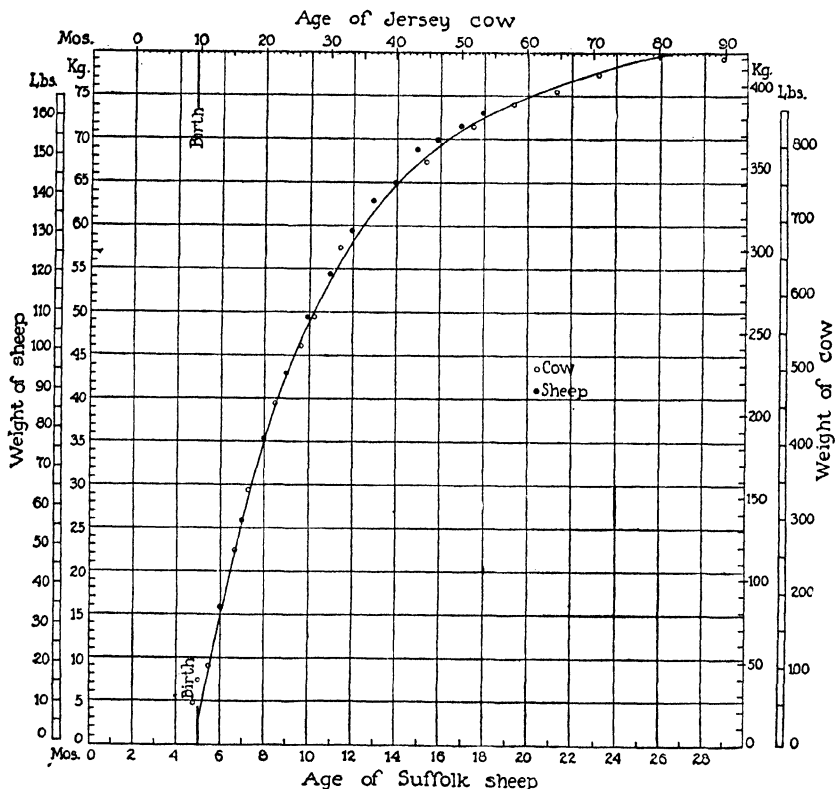

FIG. 7. Growth-equivalence of Jersey cow and Suffolk sheep. 1 month in the sheep is equivalent to 3.4 months in the cow. 1 kilo in the sheep is equivalent to 5.3 kilos in the cow.

The method of determining the mature weight, A, has been explained in the preceding communication. To calculate the age at which a given fraction of A is reached, it is only necessary to solve
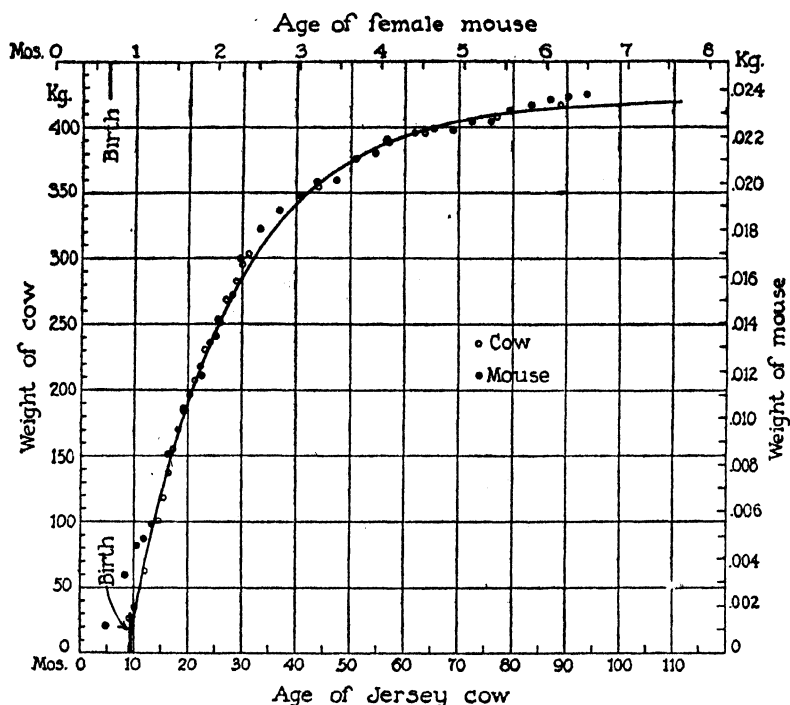
FIG. 8. Growth-equivalence of Jersey cow and female white mouse.   1 month in the mouse is equivalent (after the point of inflection) to 15.29 months in the cow.   1 gm. in the mouse is equivalent to 2.076 gm. in the cow.

The period of growth preceding the point of inflection is relatively longer in the mouse than in the cow.

for age, t, in equation (1) and substitute for the weight, W, the weight corresponding to the given fraction of A; or simply by substituting for W the given fraction of A.   Thus, from equation (1)

$$W = A - Be^{-kt}$$

$$A - W = Be^{-kt}$$

$$\log_e (A - W) = \log_e B - kt$$

$$\log_e B - \log_e (A - W) = kt$$

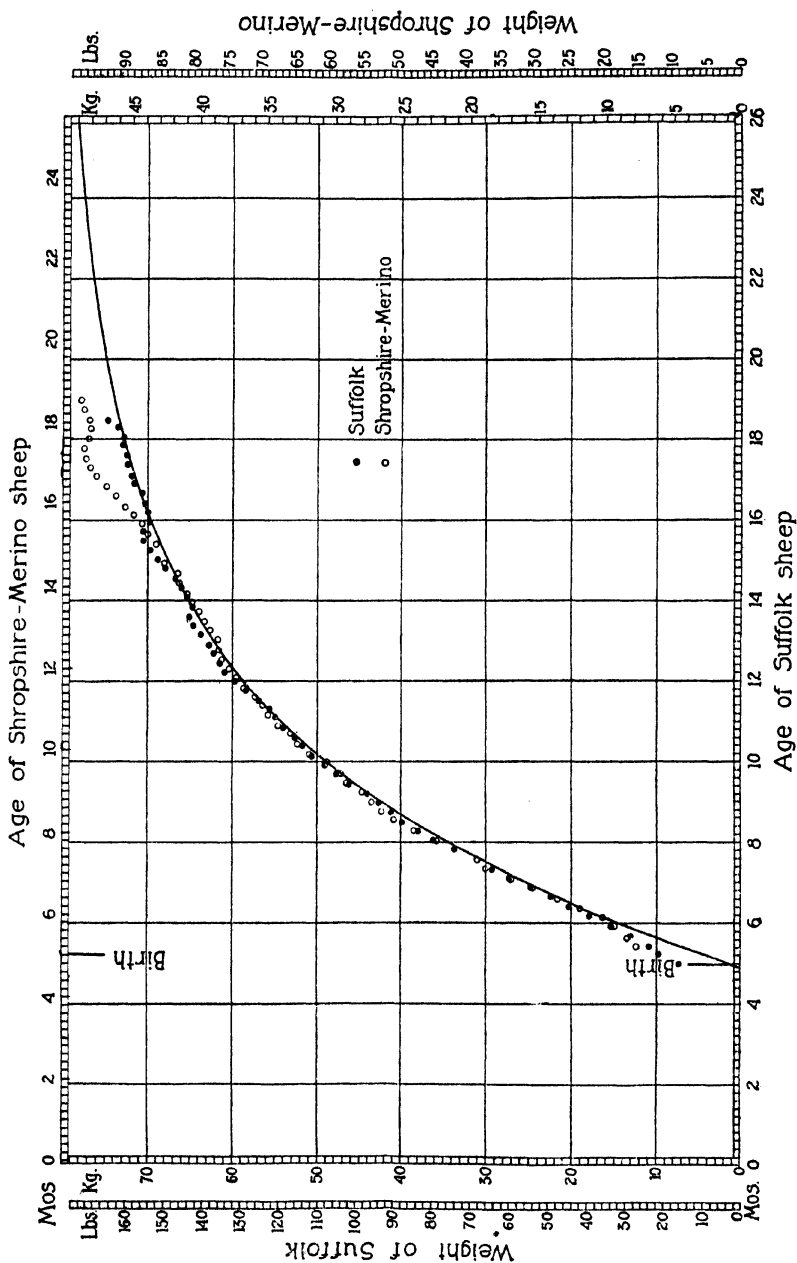$$t = \frac{\log_e B - \log_e (A - W)}{k}$$

Fig. 9. Growth-equivalence of Suffolk sheep and Shropshire-Merino sheep. A unit weight in the Shropshire-Merino sheep is equivalent to 1.6 units weight in the Suffolk sheep.

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Now, if it is desired to determine the age t when .9 of A is reached, W is substituted by .9, and we obtain

$$t = \frac{\log_e B - \log_e (A - .9A)}{k}$$

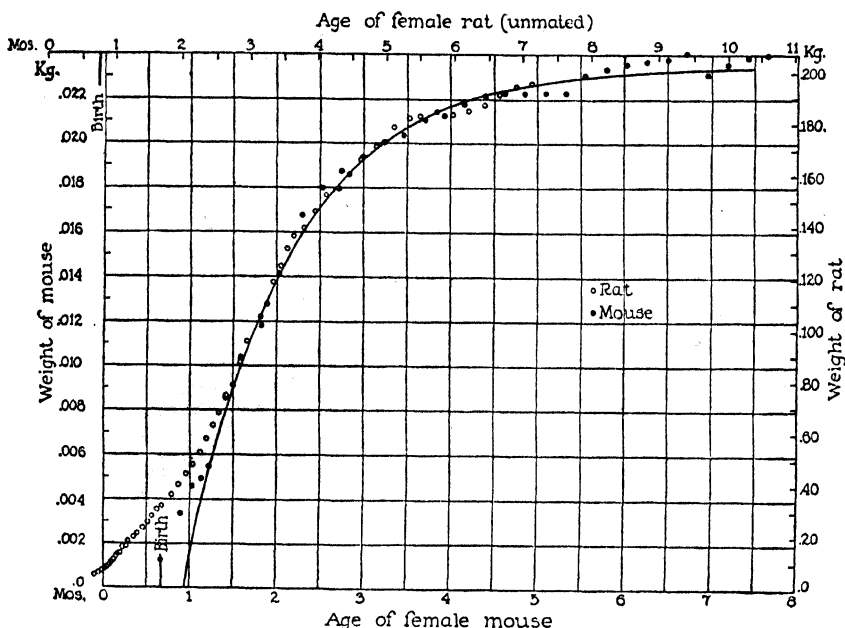$$= \frac{\log_e B - \log_e .1A}{k}$$

Fig. 10. Growth-equivalence of unmated female white rat and female white mouse. 1 month in the mouse is equivalent (after the point of inflection) to 1.27 months in the rat. 1 gm. in the mouse is equivalent to 8.64 gm. in the rat.

The period preceding the point of inflection appears to be much longer in the rat than in the mouse; *i.e.*, the infantile and juvenile periods appear to be relatively much longer in the rat than in the mouse.

Ages when different fractions of the mature weight are reached as computed from equation (3), and also the ages when different fractions of mature weight are reached as obtained by direct interpolation from the smoothed weight-age curves are shown in Table II. The agreement between these two sets of values is satisfactory for the

later stages of growth. The deviation between these two sets of values increases, however, with decreasing age due to the fact that the differences in the values of t^* make themselves felt in an increasing degree. These agreements and deviations are also shown graphically
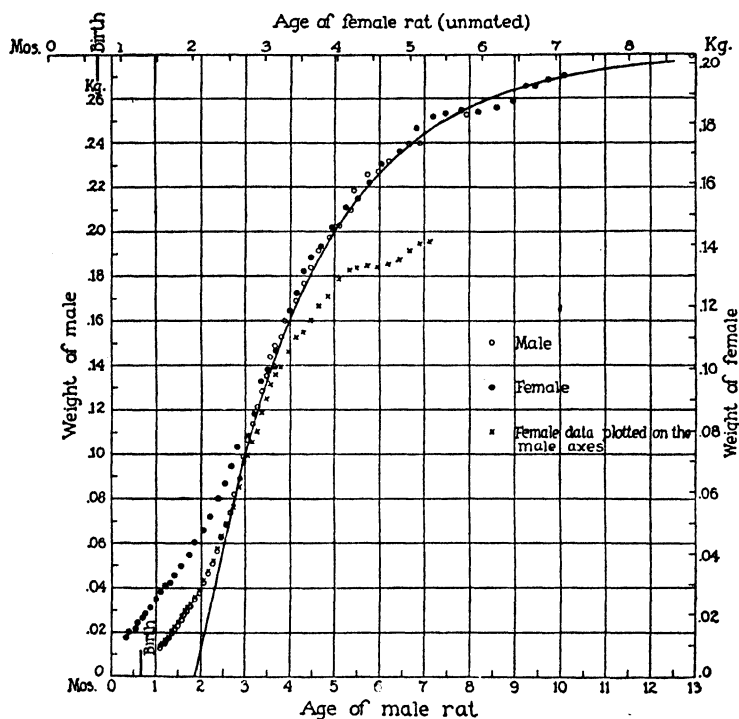

FIG. 11. Growth-equivalence of male and female white rat of Donaldson, Dunn, and Watson. After the point of inflection, 1 month in the female is equivalent to 1.61 months in the male. 1 gm. in the female is equivalent to 1.38 gm. in the male.

The period of growth following the point of inflection is evidently much longer in the male than in the female.

in Fig. 1, a. Table II is supplemented by Fig. 15 from which different fractions of the mature weight may be easily interpolated if the numerical value of k is known. (For errors involved cf. Fig. 1, a.)

Before closing this paper, it is necessary to call attention to Donaldson's method of determining equivalence of age between man and

rat on the basis of duration of life.[3] Donaldson assumed that the
rat at 3 years is comparable in age to man at 90 years, and that ap-
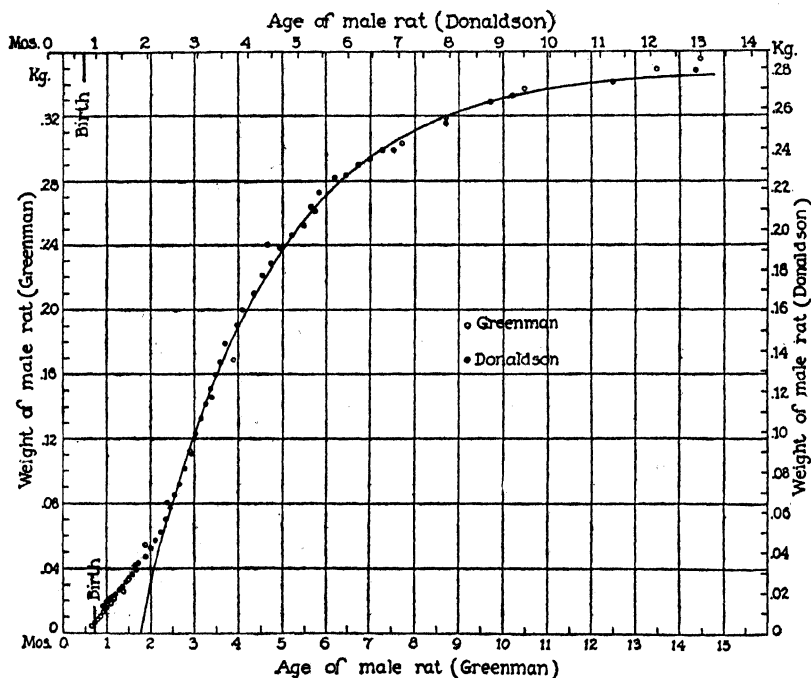proximately the same proportional relations hold for fractions of this

FIG. 12. Growth-equivalence between the "specially well cared for and fed"
albino male rats of Greenman and Duhring, and the male rats of Donaldson, Dunn,
and Watson.   1 month in the rat of Donaldson, Dunn, and Watson is equivalent
to 1.14 months in the rat of Greenman and Duhring; that is, the rat of Greenman
and Duhring had a longer period of growth.   1 gm. in the rat of Donaldson,
Dunn, and Watson is equivalent to 1.25 gm. in the rat of Greenman and Duhring.

period.   One objection against this method consists, as Donaldson
pointed out, in the incomplete information concerning duration of
life.   The objections against the assumption that the same propor-
tional age relations hold throughout life for two unrelated animal
forms have already been discussed.   It may be of some interest to

[3] Donaldson, H. H., A comparison of the white rat with man in respect to the
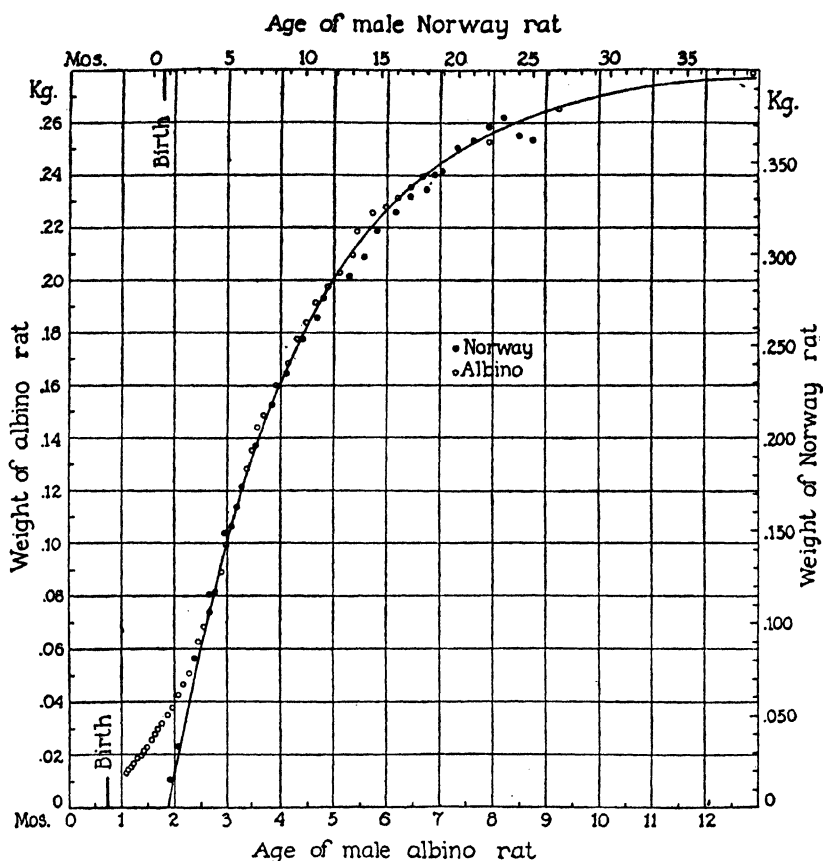growth of the entire body, Boas Anniversary Volume, New York, 1906, 5.

FIG. 13. Growth-equivalence of male Norway rat and male albino rat of Donaldson, Dunn, and Watson.

1 month in the albino rat is equivalent (after the point of inflection) to 3.3 months in the Norway rat. This is a remarkable fact.

The other remarkable fact is the relatively early and abrupt inflection in the curve of the Norway rat as compared to the albino rat.

The question naturally suggests itself, what would be the numerical values of A and k in crosses between Norway and albino rats or, more generally, how do the characters A and k behave in genetic operations; also what is the relation between A and k—can these two characters be varied independently? The conception of k should give the geneticist a quantitative measure in the study of inheritance of the character *rate* of growth, and help him in developing rapidly growing animals of large size—if such development is physiologically possible.
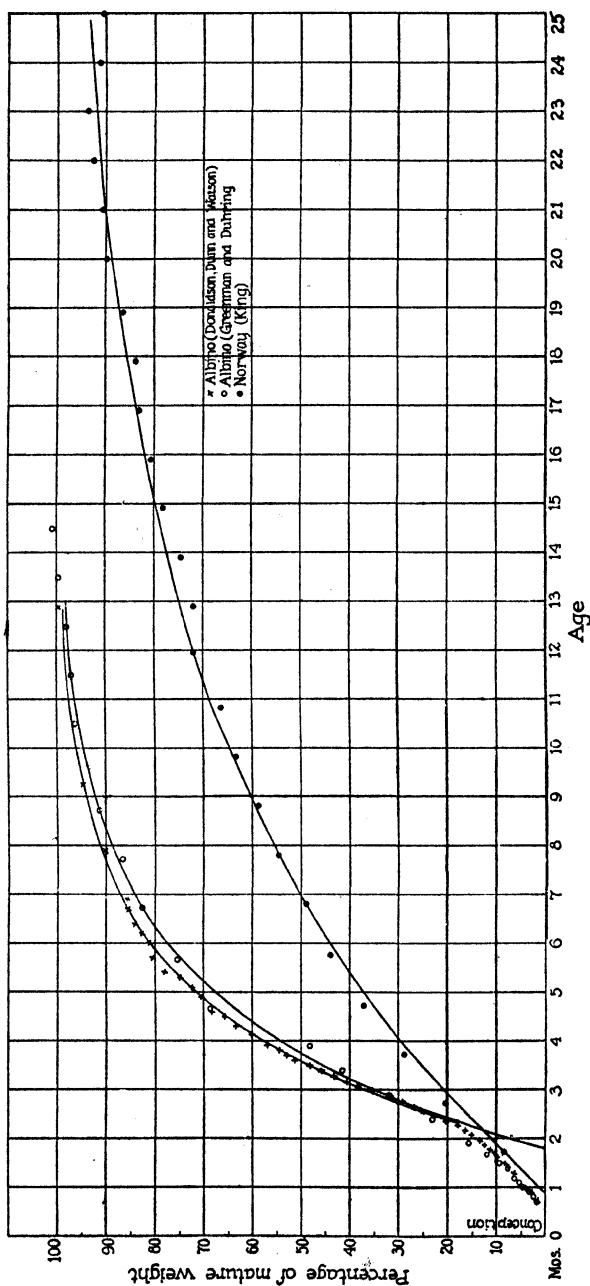
FIG. 14. Growth curves of the two sets of male albino rats and male Norway rat. The body weights are represented as percentages of the mature weight.

This chart is intended to indicate the effect of environment and domestication on the course of growth and on the shape of the growth curve of the rat.

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know that it requires about 7 months from conception to reach 98 per cent of the mature weight in the female rat, and 216 months in
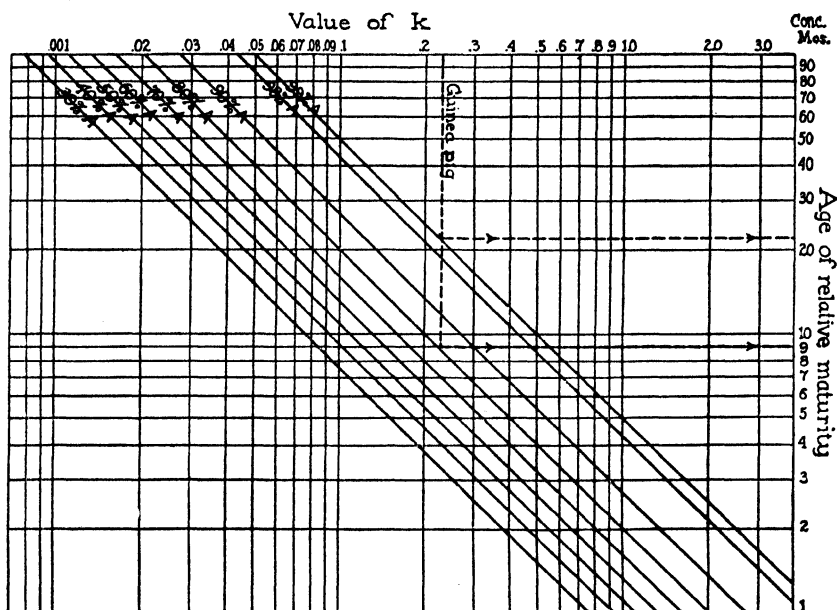

FIG. 15. (Supplementing Table II.)   The relation between the numerical value of k and the ages when different fractions of the mature weight are reached.   Thus, the value of k for the guinea pig is .23 and from the chart it is easy to read that the guinea pig reaches 99 per cent A at 22 months, 80 per cent A at 9 months (from conception) and so on.

man (*cf.* Fig. 1 of the preceding paper).   1 year in the rat corresponds to 28 years in man, which agrees within the limits of experimental error with Donaldson's estimate of equivalence.

<div align="center">SUMMARY.</div>

The numerical values of the growth constants given in the preceding paper of this series are utilized for determining equivalence of growth age and growth weight in several animal forms.   The results are presented in the form of two age-equivalence tables and fifteen age-equivalence and weight-equivalence charts.

SOURCES OF DATA.

Cattle (*Eckles*) *and sheep* (*Murray*): The numerical data were taken from *Univ. Missouri Agric. Exp. Station, Research Bull. 62*, 1923.

Swine: The averages were obtained from unpublished records by Professor F. B. Mumford, Dean of the College of Agriculture and Director of the Agricultural Experiment Station, University of Missouri, Columbia.

Guinea pig: The averages were kindly furnished to the writer by Dr. Sewal Wright, United States Department of Agriculture, Washington, D. C.

White rat: Male and female, data by Donaldson, Dunn, and Watson, in The rat, Memoirs of The Wistar Institute of Anatomy and Biology, No. 6, Philadelphia, 1915.

White rat, male: Data by Greenman and Duhring, in The rat. Data and reference tables by H. H. Donaldson, Philadelphia, 1924.

Norway rat, male: Data by King in The rat. Data and reference tables by H. H. Donaldson, Philadelphia, 1924.

Mouse: Robertson, T. B., *J. Biol. Chem.*, 1916, xxiv, 369.

THE EFFECT OF RADIOACTIVE RADIATIONS AND X-RAYS ON ENZYMES.

V. The Influence of Variation of the Thickness of the Absorbing Layer of Solutions of Pepsin upon the Rate of Radiochemical Inactivation of the Enzyme.

By RAYMOND G. HUSSEY and WILLIAM R. THOMPSON.

(From the Brady Laboratory of Pathology and Bacteriology, Yale University School of Medicine, New Haven, and the Douglas Research Laboratory, Memorial Hospital, New York.)

Some time ago we commenced an investigation of the effect of the radiations from the radioactive products in equilibrium with radium emanation upon enzymes in solution. In our first experiments we irradiated dilute solutions of trypsin and found that definite inactivation resulted. We succeeded in following the course of this radiochemical change and found that the rate of change in the logarithm of the concentration of active enzyme was proportional to the power of the radioactive source. The experimental arrangement employed in these experiments had many practical disadvantages and in addition it was not suitable to the study of the efficiency of power utilization in the irradiated system. A satisfactory method of procedure was found in the use of a spherical glass flask to contain the solution to be irradiated with the source of radiation in a smaller spherical glass bulb at its center. Under these conditions of irradiation the course of inactivation of trypsin[1] was followed with results in agreement with those found in the original experiments.[2] Our observations were then extended to a study of the radiochemical inactivation of pepsin[3] and invertase[4] where we observed results that were in agreement with those found in the case of trypsin.

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[1] Unpublished experiments.

[2] Hussey, R. G., and Thompson, W. R., *J. Gen. Physiol.*, 1922–23, v, 647.

[3] Hussey, R. G., and Thompson, W. R., *J. Gen. Physiol.*, 1923–24, vi, 1.

[4] Hussey, R. G., and Thompson, W. R., *J. Gen. Physiol.*, 1925–26, ix, 211.

With these facts established we were in a position to study other important aspects of this type of radiochemical change. Our attention was directed to the consideration of the effect of varying the thickness of the liquid layer upon the value of the mean speed coefficient (k).

$$\frac{dQ}{dt} = - kQP \qquad (1); \quad \text{or} \quad \frac{d \log Q}{dt} = - kP \qquad (2)$$

where Q is the concentration of active enzyme (expressed in arbitrary units) at the time, t (expressed in hours from the start of the irradiation), and P is the power of the source at that same instant. The relation between these variables may be variously expressed as was indicated in a previous communication.[5] A convenient form of expression for the purpose of computing the value of k is as follows:

$$k = \frac{1}{W} \log_e \frac{Q_0}{Q}$$

where $W = \int_0^{^\circ t} P\, dt$ and Q_0 is the initial concentration of active enzyme.

As has been shown by our previous observations, when the volume of the irradiated system (in the spherical arrangement described above) is constant, the value of the speed coefficient is also constant. As this relation holds for any of the enzymes studied we are at liberty to choose any one of them for the present purpose. Our first attempt was made with invertase as a matter of convenience. We found that over a certain range of variation the product of the values of the speed coefficient obtained and the volume of solution irradiated was approximately a constant.[4] Unfortunately, the determination of speed coefficients at small volume could not be made satisfactorily with the invertase preparation then available. The results obtained indicated that the effect of the gamma radiation was negligible with respect to that of the beta radiation; but, however, from these results no definite information could be obtained in regard to what might be considered the lower limit of liquid layer for sensibly complete absorption of beta radiation by the radiosensitive system. Since this phase of our

[5] Hussey, R. G., and Thompson, W. R., *J. Gen. Physiol.*, 1923–24, vi, 7.

study is of considerable importance to our investigation as a whole we have employed pepsin in an attempt to gain this information and to confirm the results obtained with invertase. It is the purpose of this communication to present the results obtained.

Experimental Procedure.

The pepsin solution was prepared by dissolving 3.125 gm. of granular pepsin in distilled water containing 2.50 cc. of 0.1 M HCl, diluting to 250 cc. in a volumetric flask with distilled water, filtering, and add-

TABLE I.

Pepsin solution irradiated. Volume (V).	Thickness of layer (d).	Curie-power hours. (W)	Units of active pepsin. (Q)	$k = \dfrac{1}{W} \log_e \dfrac{Q_0}{Q}$	$K = kV$
cc.	*cm.*		$Q_0 = 2.913 \pm 0.015$		
18.34	1.419	23.22	1.934 ± 0.023	0.0177	0.324
11.88	1.199	18.03	1.886 ± 0.012	0.0289	0.343
8.12	1.031	10.29	1.947 ± 0.017	0.0392	0.319
4.59	0.816	5.811	2.024 ± 0.017	0.0627	0.288
2.83	0.661	3.572	1.985 ± 0.036	0.108	0.304
1.26	0.454	1.600	2.262 ± 0.027	0.158	0.199
1.26	0.454	1.600	2.207 ± 0.029	0.174	0.218

The precision measure of Q is the a. d. The values of Q given are in each case the mean of eight determinations with the exception of the two results for the volume 1.26 cc. where the value given is the mean of four determinations. The value of Q_0 is the mean of 40 determinations made at different times distributed over the course of the experiment. The power of the radioactive source in these experiments lay between 550 and 50 curie-powers.

ing a crystal of Merck's Reagent Thymol to the filtrate. The pH of this solution was found to be 4.4 by colorimetric measurement. The stock (control) solution was kept in a pyrex flask (painted with asphaltum on the outside) in a thermostat at 10.0°C. Irradiations were performed in another thermostat regulated to 10.00±0.03°C. That the same form of curve is obtained for the radiochemical inactivation of pepsin at 10°C. as had been found at 0°C. was shown by a short series of irradiations, the results of which are given in another communication.[6]

[6] Hussey, R. G., and Thompson, W. R., *J. Gen. Physiol.*, 1925–26, ix, 315.

The volumes of the spherical irradiation flasks used in the experiments were such that when the small radium emanation bulb (previously described[4]) was in position at their center they would contain 18.34, 11.88, 8.12, 4.59, 2.83, and 1.26 cc. respectively. Pipettes were made to deliver these volumes of solution, and calibrated gravimetrically. Obviously, when solution was placed in these flasks and the radium emanation bulb inserted in the center, the solution assumed the form of a spherical shell. The thickness (d) of this spherical shell has been previously referred to as the thickness of the liquid layer. It may be computed for any given irradiation flask from a knowledge of the outside diameter ($2a$) of the radium emanation bulb (in this case 0.432 cm.) and the volume of the spherical shell (V). The calculated values of d for the flasks mentioned above were 1.419, 1.199, 1.031, 0.816, 0.661, and 0.454 cm. respectively. The results of a series of irradiations with these flasks of pepsin solution at 10°C. is given in Table I where the values of the speed coefficient (k) are given and in addition the product of this value and the volume (V) of solution irradiated ($K = kV$).

The method of determining the active pepsin concentration was the same as we have employed in our previous experiments.[3]

<center>DISCUSSION.</center>

As has been indicated in the paper previously referred to,[4] when the thickness of the liquid layer is sufficient to absorb practically all of the beta radiation, and the speed of diffusion is great enough to maintain uniform concentration of enzyme throughout the liquid system, and in addition the effect of the gamma radiation is negligible; it would be expected that the mean speed coefficient of the reaction would vary inversely with the volume (V), i.e.

$$- \frac{d \log Q}{P \, dt} = - \frac{d \log Q}{dW} = k = \frac{K}{V} \tag{5}$$

where K is constant over this range.

It will be observed in Table I that the values of the product kV are not significantly different when the thickness of liquid layer (d) lies between 0.661 and 1.419 cm.; i.e., the differences are not significant

with respect to the precision measure. The region explored with invertase lay within this range (0.816 to 1.419 cm.) where satisfactory agreement was obtained between the values of K. Our present results, therefore, serve as a confirmation of those obtained with invertase. However, the values of kV obtained where $d = 0.454$ cm. do differ significantly from the others. If we assume that in this case the speed of diffusion was not significantly changed, and it seems reasonable to make this assumption, it follows that the fraction of the available energy absorbed by the system was decreased. Indeed, it may be remarked that a suggestion of a drift appears in the data in the direction of decreasing values for K with decreasing values of d as the value of d approaches 0.454 cm.; but this drift (it appears in the observations on invertase also[4]) cannot be considered as significant without the evidence of additional data, although it is in keeping with what might be expected, and approximately with what was anticipated at the beginning of these studies. It is suggested, furthermore, that, given proper experimental conditions, K may be employed as a measure of the absorbing power[7] of an irradiated system.

CONCLUSION.

Evidence is presented which indicates: (1) that the effect of gamma radiation is negligible with respect to that of beta radiation upon pepsin in dilute solution under the conditions employed in the experiments made; (2) approximately the thickness of fluid layer which may be regarded as necessary and sufficient to practically completely absorb the available beta radiation; (3) that the mean reaction speed coefficient in radiochemical inactivation of pepsin varies inversely with the volume of solution irradiated if the thickness of the fluid layer satisfies the sufficient condition stated in (2), and beyond this as far as has been studied.

[7] Duff, A. W., A text-book of physics, Philadelphia, 4th edition, 1916, 641.

THE EFFECT OF RADIOACTIVE RADIATIONS AND X-RAYS ON ENZYMES.

VI. THE INFLUENCE OF VARIATION OF TEMPERATURE UPON THE RATE OF RADIOCHEMICAL INACTIVATION OF SOLUTIONS OF PEPSIN BY BETA RADIATION.

BY RAYMOND G. HUSSEY AND WILLIAM R. THOMPSON.

(*From the Brady Laboratory of Pathology and Bacteriology, Yale University School of Medicine, New Haven, and the Douglas Research Laboratory, Memorial Hospital, New York.*)

We have observed that trypsin,[1] pepsin,[2, 3] and invertase[4] in dilute solutions are inactivated when irradiated by the radiations from the radioactive products in equilibrium with radium emanation. We have presented evidence which indicates that the inactivation is effected by the beta radiations from the radioactive source.[3] This radiochemical inactivation of the enzymes named has been found to proceed as a monomolecular radiochemical reaction.

$$\frac{dQ}{dt} = -kQP \qquad (1)$$

where Q is the concentration of active enzyme (in arbitrary units) at the time (t), expressed in hours, P is the power of the radioactive source at the same instant, and k is the speed coefficient of the reaction. The reader is referred to another paper[5] in which this equation and its implications are discussed at length.

The irradiations referred to above were made at the temperature of melting ice. Recently we have observed the effect of the same radia-

[1] Hussey, R. G., and Thompson, W. R., *J. Gen. Physiol.*, 1922–23, v, 647.

[2] Hussey, R. G., and Thompson, W. R., *J. Gen. Physiol.*, 1923–24, vi, 1.

[3] Hussey, R. G., and Thompson, W. R., *J. Gen. Physiol.*, 1925–26, ix, 309.

[4] Hussey, R. G., and Thompson, W. R., *J. Gen. Physiol.*, 1925–26, ix, 211.

[5] Hussey, R. G., and Thompson, W. R., *J. Gen. Physiol.*, 1923–24, vi, 7.

tions on pepsin in dilute solution at 10°C. It is the object of this paper to present these results (Table I) together with other observations made with the object of ascertaining what effect temperature variation has upon the value of the speed coefficient (Table II). The technical procedure was otherwise the same as that employed in the experiments reported in the paper immediately preceding this one.[3]

TABLE I.

Radiochemical Inactivation of Pepsin in Dilute Solution.

Volume irradiated, 4.59 cc. Temperature, 10°C.

Curie-power hours. (W)	Units of active pepsin. (Q)	$k = \frac{1}{W} \log_e \frac{Q_0}{Q}$ $k \times 10^2$
	$Q_0 = 2.681$	
3.247	2.144	6.89
5.930	1.753	7.11
11.32	1.106	7.82

TABLE II.

Effect of Variation in Temperature on the Radiochemical Inactivation of Pepsin in Dilute Solution.

Volume irradiated, 4.59 cc.

Temperature.	Curie-power hours. (W)	Units of active pepsin. (Q)	$k = \frac{1}{W} \log_e \frac{Q_0}{Q}$ $k \times 10^2$
°C.		$Q_0 = 2.681$	
0	7.650	1.693	6.01
0	8.024	1.592	6.49
20	5.930	1.715	7.53
20	5.930	1.727	7.42

The data presented in Table I indicate that the course of radio-chemical inactivation of pepsin solution at 10.0°C. may be represented by a curve of the same form as that at 0.0°C.[2] The data presented in Table II for 0.0°C. and at 20.0°C. together with those in Table I for 10.0°C. indicate what influence temperature variation may be expected to have upon the speed of the reaction. However, no attempt is made, at the present time, to establish definite values for *k* and

its temperature coefficient which will hold precisely for any source of radiation of the type described, inasmuch as definite standardization of the thickness of the glass wall of the bulb containing the radium emanation has not been possible up to the present time. Consequently, experiments made with different bulbs should not be expected to yield precisely the same reaction speed coefficients as these have been calculated with respect to the power of the radioactive source and not with respect to what fraction of this is available to the radiosensitive system. In an attempt to compare the reaction speed coefficients published by us in various communications this fact must be borne in mind.

CONCLUSION.

Data are presented which indicate that variation in temperature is associated with only slight variation in the speed of the radiochemical inactivation of pepsin in dilute solution.

CARBON DIOXIDE PRODUCTION AND DURATION OF LIFE OF DROSOPHILA CULTURES.

BY JOHN H. NORTHROP.

(From the Laboratories of The Rockefeller Institute for Medical Research.)

One of the most concrete and plausible mechanisms which have been proposed for the regulation of the duration of life is what may be termed the *energy limit*, elaborated by Rubner.[1]   Rubner stated that the total energy transformed per kilo of body weight during the total life of the animal was approximately constant for a large number of animals.   He suggested, therefore, that the duration of life of the individual was determined by the time required to transform this quantity of energy.   Slonaker[2] found that 4 albino rats which were allowed to exercise freely died sooner than others which were allowed only limited exercise, and this has been considered by Pearl[3] as additional evidence in favor of Rubner's view.   It was found by Loeb and the writer[4] that the duration of life of aseptic *Drosophila* cultures was a function of temperature, the insects living longer at a low temperature.   Since they are also more sluggish at a lower temperature Pearl has suggested that this also is evidence in favor of the energy limit. There is further confirmation of the idea in the fact, as pointed out by Crozier,[5] that the temperature coefficients of the duration of life and of the rate of oxidation may be similar.   No direct measurements comparing the total energy production with the duration of life have been made, and the present experiments with *Drosophila* were designed to test this point.   It has been found that there is considerable variation in the total amount of CO_2 produced by cultures of *Drosophila*

[1] Rubner, M., Das Problem der Lebensdauer, München and Berlin, 1908.

[2] Slonaker, J. R., *J. Animal Behavior*, 1912, ii, 20.

[3] Pearl, R., The biology of death, Monographs on experimental biology, Philadelphia and London, 1922, 213.

[4] Loeb, J., and Northrop, J. H., *J. Biol. Chem.*, 1917, xxxii, 103.

[5] Crozier, W. J., *J. Gen. Physiol.*, 1924–25, vii, 189.

during their entire life under different conditions, and that, therefore, the duration of life of the insect is not determined by the time required to transform a definite amount of energy.

Methods.

The flies used in these experiments were taken from the 195th to the 205th generation of aseptic cultures used by Loeb and the writer.[4] These flies have been inbred from the original pair of aseptic flies; the cultures have been kept in the dark at room temperature and raised on sterilized yeast. It will be noted that the duration of life differs markedly from that given previously. A progressive decrease
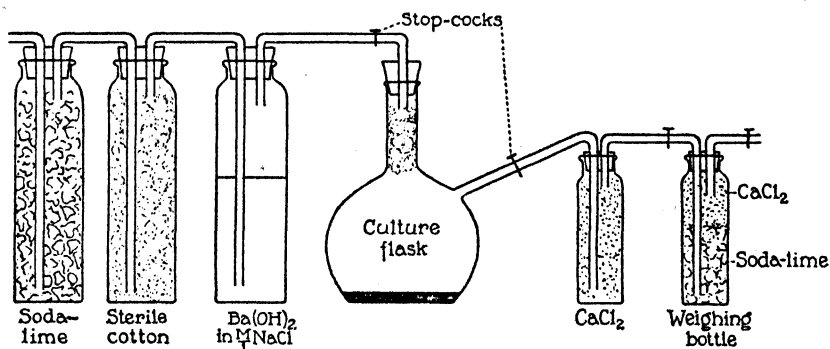

FIG. 1. Apparatus for CO₂ determination.

in the duration of life of these cultures, especially at low temperatures, had been noted for several years.

Determination of CO₂.

The arrangement of the apparatus for the determination of CO_2 is evident from Fig. 1. The cultures were connected to a soda-lime weighing tube as shown in the figure. A slow current of air was blown through the flask every 2 or 3 days for 3 hours and the CO_2 weighed. The number of larvæ, pupæ, or imagos was also counted, and the CO_2 produced per 100 individuals was calculated. A control flask containing no insects, run simultaneously, showed no increase in CO_2. The culture flasks themselves were also continued after all the insects were dead. After the death of the insects no CO_2 was found, showing

that the apparatus was air-tight and that the CO_2 in the air stream had been completely removed by the soda-lime. The larvæ were grown on sterilized yeast as already described and the imagos were kept on glucose agar. Any flasks showing contamination with microorganisms were discarded.

The 15°, 26°, and 30°C. cultures were kept in the dark in water-jacketed incubators, and the 22–26°C. light culture was kept at the room temperature, in diffuse daylight, and in addition illuminated with a 40 watt bulb attached to a circuit breaker so that the light was turned on and off at irregular intervals. This culture was also disturbed more or less by persons working in the laboratory and the flies were much more active than those in the dark. Each culture contained from 300 to 400 flies.
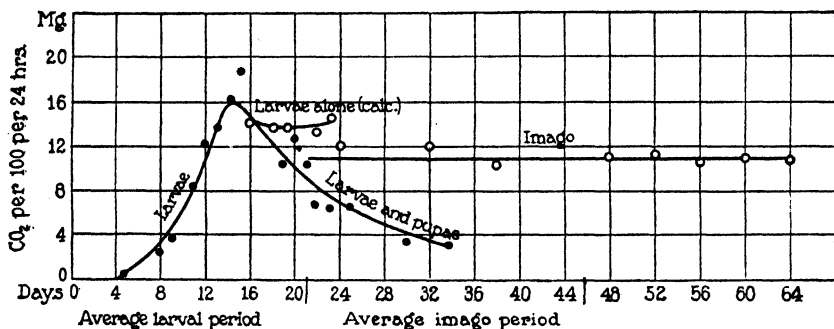

FIG. 2. Production of CO_2 per 100 individuals per day, in dark, at 16°C.

The result of a complete experiment is shown in Fig. 2, in which the CO_2 per 24 hours per 100 individuals has been plotted against the time in days. The curve confirms the results of Fink,[6] in that it shows a rapid increase during the larval period followed by a decrease in the pupal period. Since the curve is per individual and not per unit of weight, the rise is sharper than in the CO_2 per unit weight curve as it includes the increase in size of the larvæ. It will be noted that the curve of CO_2 production of the imagos is practically constant throughout life. This result was obtained in every experiment, the only decrease noted being at the end of the experiment when the number of

[6] Fink, D. E., *J. Gen. Physiol.*, 1924–25, vii, 527.

TABLE I.

Duration of Life and CO$_2$ Production of Drosophila Cultures under Various Conditions.

Larvae.

Experiment.	Temperature, etc.	Average duration of larval period. (days)	Average CO$_2$ per 24 hrs. per 100. (mg.)	Total CO$_2$ per 100 larvae. (mg.)
1/8	15 Dark.	21	8.0	170
3/17	15 "	20	7.5	150
2/19	26 "	6.3	13.0	83
2/19a	26 "	6.1	13.4	82
1/9	26 "	6.2	12.0	73
1/9	22–26 Light.	6.5	10.8	70
2/11	22–26 "	7.2	12.0	87
2/19	22–26 "	6.6	17.0	112 } 94
2/19	22–26 "	7.3	15.0	108
3/16	30 Dark.	4.6	14.0	66
2/19	30 "	6.1	15.4	94

Imagos.

Experiment.	Temperature, etc.	Average duration of life. (days)	Average CO$_2$ per 100 imagos per 24 hrs. (mg.)	Total CO$_2$ per 100 imagos. (mg.)	Total CO$_2$ for entire duration of life. (mg.)
2/10	16 Dark.	24	11.4	275	445
4/15	16 "	26	11.1	290	440
4/29	26 "	13	14.5	189	
5/17	26 "	9.3	12.0	110 } 192	272
4/15	26 "	9.0	29.0	260	
5/17	26 "	9.2	23.0	210	
3/2a	22–26 Light.	15.0	22.0	330	
3/2b	22–26 "	16.0	21.0	331 } 317	411
3/31	22–26 "	15.2	18.0	275	
3/31b	22–26 "	15.0	22.0	330	
3/30	30 Dark.	7.6	19.6	149	
2/26	30 "	8.1	17.5	141 } 166	246
3/9	30 "	11.9	17.6	210	

flies was very small and the error proportionately large. There is, therefore, no evidence of any "running down" as might have been expected were the duration of life determined by the transformation of a limiting amount of energy.

A summary of all the experiments is given in Table I. The experiments show that the total amount of CO_2 produced by the insects during the larval, the imaginal, or during the entire duration of life varies considerably with the conditions.[7] More CO_2 is produced at 15° than at 26° when both cultures are in the dark; *i.e.*, the temperature coefficient of CO_2 production is smaller than that for the duration of life or the duration of the larval period. The cultures which were exposed to the light, however, produced much more CO_2 than those in the dark.[8] This effect of light on CO_2 production is well known, and was shown by Loeb[9] to be due to an increase in muscular activity, since insect pupæ are not affected. At 30°C., in the dark, there is a still further decrease in the total CO_2, owing to the fact that the CO_2 per day remains nearly constant while the rate of growth and duration of life is shortened. 30°C. is above the normal temperature range of the insect since successive generations cannot be reared at this temperature, and it is possible that the results at this temperature are not significant on this account, since it is evident that death due to injury cannot be determined by energy limitations.

The results are corroborated by the fact that quite high light intensities have no effect on the duration of life of these insects,[10] whereas numerous investigators have shown that illumination markedly increases the CO_2 production.

SUMMARY.

The total CO_2 produced by aseptic *Drosophila* cultures during the entire duration of life has been determined at 15°, 26°, and 30°C. in the dark and at 22–26°C. in the light.

[7] Owing to experimental difficulties the CO_2 production of the pupæ has been omitted. It is very small compared to that of either larvæ or imagos.

[8] The longer duration of life of the light cultures is due to the slightly lower temperature.

[9] Loeb, J., *Arch. ges. Physiol.*, 1888, xlii, 393.

[10] Northrop, J. H., *J. Gen. Physiol.*, 1925–26, ix, 81.

The total amount of CO$_2$ produced is not constant but is greater at 15° than at 26° or 30°, and is much greater in the light than in the dark.

The total duration of life, therefore, is not determined by the time required to produce a limiting amount of CO$_2$.

THE EFFECT OF ADVANCE IN LACTATION AND GESTA-
TION ON MAMMARY ACTIVITY.

By W. L. GAINES AND F. A. DAVIDSON.

(From the Department of Dairy Husbandry, University of Illinois, Urbana.)

This study is based on certain of the records of Guernsey cows as published in the Advanced Register of the American Guernsey Cattle Club.[1] This extract is presented in the interest of the physiological bearing of the data on the problems of milk secretion. The records are unique in comparison with the natural course of events in the wild state, in that the rate of mammary activity has been artificially developed to a point far in excess of the requirements of the young. Also, in many cases, the recurrence of pregnancy is unusually delayed and lactation is greatly protracted under the stimulation of artificial milking and liberal feeding. Similar data have been studied by Brody, Ragsdale, and Turner in this journal.[2] Other literature references are here omitted.

By appropriate statistical treatment a series of eleven observed values was obtained for farrow cows, each value representing the yield for a month, the mid-point of which was 1, 2, ... or 11 months after calving. Twelve additional similar series were calculated, one each for cows conceiving .5, 1.5, ... and 11.5 months after calving.

Milk yield is regarded as the result of the rate of milk secretion. The rate of milk secretion is treated as continually decreasing at a

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[1] The results are being published in detail as a bulletin of the Illinois Agricultural Experiment Station (Gaines, W. L., and Davidson, F. A., *Illinois Agric. Exp. Station, Bull. 272*, 1926 (in press).

[2] Brody, S., Ragsdale, A. C., and Turner, C. W., *J. Gen. Physiol.*, 1922–23, v, 441, 777. Brody, S., Turner, C. W., and Ragsdale, A. C., *J. Gen. Physiol.*, 1923–24, vi, 541.

rate proportional to its momentary value, in accordance with the equation:

$$\frac{dy}{dt} = ae^{-kt} \tag{1}$$

in which y = yield in pounds, t = time in months from calving, a is a constant representing the initial rate of yield in pounds per month, and k is a constant representing the rate of decrease per month in the rate of yield per month.

Equation (1) was applied to the observed values as:

$$y_m = Ae^{-kt} \tag{2}$$

in which y_m is the yield for a month, time being reckoned to the middle of the month; and $A = a\dfrac{e^{.5k} - e^{-.5k}}{k}$. Practically, for the values of k at present involved, A and a may be considered as equal.

Cows Farrow or in Early Pregnancy.

The data for farrow cows and the curves from equation (2) fitted by the method of least squares are shown in Fig. 1. The lower series of observations and curve relate to milk yield. The upper series of observations and curve relate to fat yield. The middle series needs a word of explanation. It is an estimate of the energy value of the milk solids yield, expressed in terms of normal milk of 4 per cent fat content, and designated F.C.M., fat corrected milk. The values are derived from the milk (M) and fat (F) by the equation $F.C.M.$ = $.4M + 15F$ (1 pound $F.C.M.$ = 331 large calories.) As a quantitative measure of mammary activity, energy yield has several biological arguments in its favor.

It is evident from Fig. 1, that the theoretical curves correspond well with the observed values. The root mean square errors are: *fat corrected milk*, 5.23; *fat*, .303; *milk*, 7.14. Weighting the errors by their respective $1/A$'s and taking the weighted F.C.M. error as 100, the fat error is 138, and milk, 147.

Equation (2) was found applicable to the groups bred 11.5, 10.5, 9.5, 8.5, 7.5, and 6.5 months after calving. Data for the latter group are presented in Fig. 2. Pregnancy is 4.5 months advanced at the

last observation in this group.   It appears, therefore, that for the first 5 months of the gestation period pregnancy does not appreciably affect the rate of milk secretion.   Both the A and k constants of
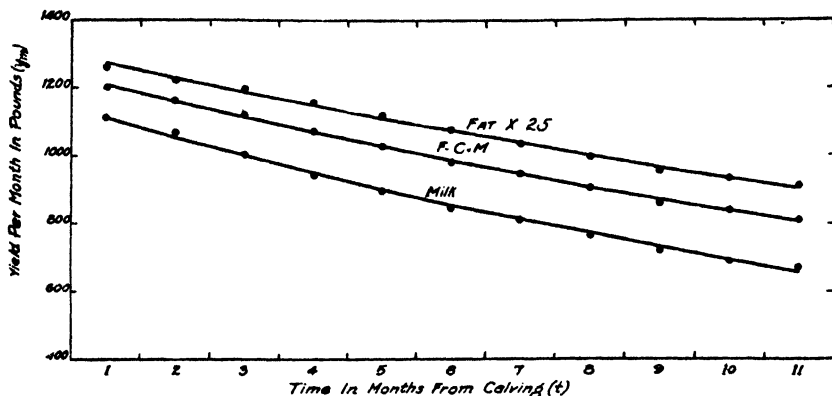

FIG. 1. Rate of activity of the mammary gland in farrow cows as measured by fat yield ($y_m = 52.575e^{-.034202t}$); milk yield ($y_m = 1167.6e^{-.052347t}$); and F.C.M. yield ($y_m = 1254.7e^{-.040524t}$).
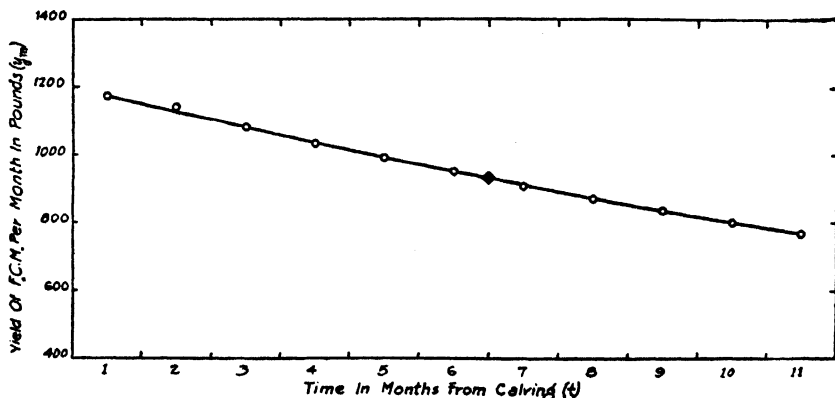
FIG. 2. Rate of activity of the mammary gland in pregnant cows as measured by F.C.M. yield ($y_m = 1220e^{-.042332t}$). Time of conception is marked by ◆ on the curve.   Up to 5 months pregnancy does not appreciably affect the rate of milk secretion.

Fig. 2 differ from those of Fig. 1 in the direction of a decrease in yield, but there is no apparent reason to connect this decrease in yield with the occurrence of pregnancy in Fig. 2.

Equation (1) furnishes a ready means of computing the relative yields for various portions of the lactation. The ratio between any two portions is a function of k. The average value found for k was .04412. Breeders of dairy cattle are particularly interested in the relation between the yield for 305 days and 365 days. Starting at the same time after calving the 305 day yield is 86.97 per cent of the 365 day yield, for the value of k given.

Cows in Advanced Pregnancy.

When pregnancy becomes further advanced in the observed lactation, equation (1) is not sufficient to describe the rate of milk secretion throughout. It is necessary to add a term to take care of the effect of pregnancy. The equation used for the groups bred 5.5, 4.5, 3.5, 2.5, 1.5, and .5 months after calving in its differential form is:

$$\frac{dy}{dt} = ae^{-kt} - be^{K(t-c)} \tag{3}$$

and as applied to the observed values:

$$y_m = Ae^{-kt} - Be^{K(t-c)} \tag{4}$$

In equations (3) and (4) the minus term measures the effect of pregnancy on milk secretion; b is a constant representing the initial rate of inhibition of the rate of milk secretion; $B = b\,\dfrac{e^{.5K} - e^{-.5K}}{K}$; K is a constant representing the rate of change per month in the rate of inhibition; c is the number of months from calving to conception, and $t - c$ is time in months from conception.

Fig. 3 shows the observed values and fitted curve of equation (4) for the group of cows bred 1.5 months after calving. In the six groups for which equation (4) was used the K constant is the same (1.09861) in each case. The B constant is also the same (.01206) in five of the six cases. Representing the inhibition of milk secretion in pounds per month by i, and time in months of pregnancy by p we have $\dfrac{di}{dp} = .01147e^{1.09861p}$. The gestation period in the cow is 9.2 months and integration between the limits 0 and 9.2 gives 256 pounds F.C.M.

as representing the average total decrease in yield due to carrying the calf full term.

The groups of cows bred 1.5 and 2.5 months after calving were each further separated into four age classes. Equation (4) applied to these age data led to the same constant value of K. The values of B varied, however, and were roughly proportional to A. That is, the decrease in yield associated with pregnancy tends to be proportional to the productive level.

Inhibition of Milk Secretion Due to Pregnancy.

Two explanations have been offered for the decrease in milk yield associated with pregnancy: one, that it is due to the demands of
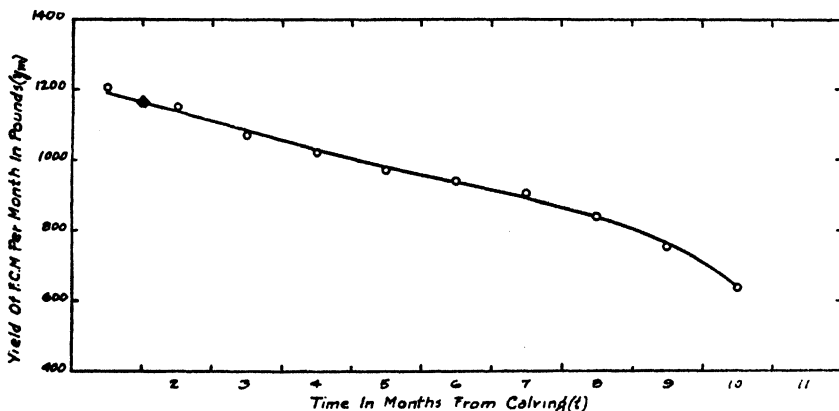

FIG. 3. Rate of activity of the mammary gland in pregnant cows as measured by F.C.M. yield ($y_m = 1250e^{-.04796t} - .01206e^{1.09861\,(t-1.5)}$). Time of conception is marked by ◆ on the curve. Advanced pregnancy occasions a marked decrease in the rate of milk secretion, measured by the minus term of the equation.

the growing fetus for nutrients; the other, that it is due to a hormone thrown into the circulation during pregnancy and acting as an inhibitor to milk secretion. The latter view seems to have the most adherents and seems to be better supported by experimental evidence. The inhibition theory leads also to a possible explanation of the increasing rate of milk secretion for a short time following parturition.

It is known that the rate of milk secretion increases following partu-

rition for a period of 10 to 30 days, or longer in individual cases. Brody, Turner, and Ragsdale have applied the equation

$$M = Ae^{-k_1 t} - Be^{-k_2 t} \tag{5}$$

to observed daily milk yields following parturition and obtained a satisfactory agreement (M = milk yield, t = time from parturition).
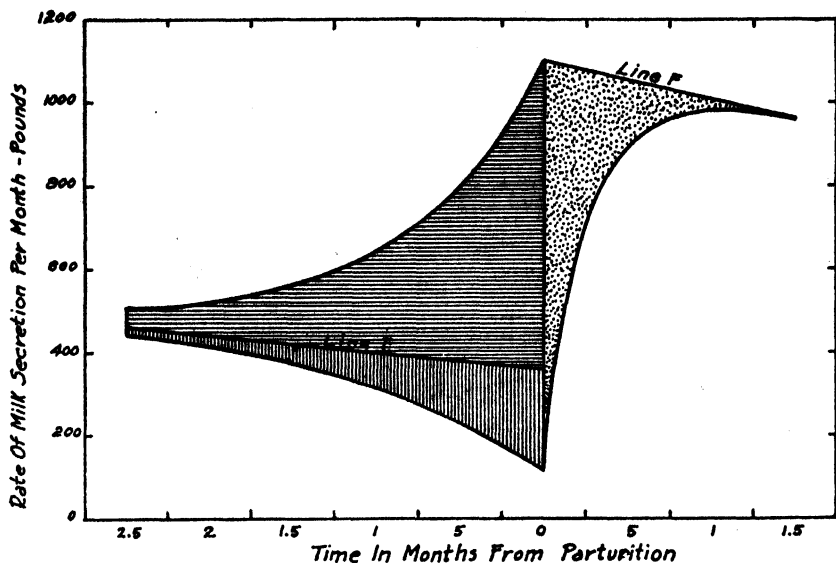

FIG. 4. Diagram illustrating the potential rate of milk secretion (upper line) and the realized rate of milk secretion (lower line) shortly preceding and following parturition. The area under the upper curve represents potential yield; the shaded area, the inhibition of pregnancy; and the area under the lower curve, the realized yield.

If pregnancy is terminated before full term the maximum rate of milk secretion following would be lowered according to the degree of prematurity. This is in accord with observed milk yields following abortion. The higher the maximum realized rate of milk secretion following parturition, the more delayed its appearance. This also is in accord with observations.

Further explanation of diagram in text.

They have interpreted their results as indicating an underlying consecutive chemical reaction as directly or indirectly responsible for the rate of milk secretion.

An alternative interpretation of the data is suggested by the in-

hibition hypothesis. This is illustrated diagrammatically in Fig. 4. The figure covers a period from 2.5 months preceding to 1.5 months following parturition. The ordinates of the lines F, represent the rate of milk secretion outside the influence of pregnancy according to equation (1). The vertically shaded portions of the ordinates represent the observed decrease in rate of yield of pregnant cows as compared with farrow cows. The horizontally shaded portions represent an assumed increase in the potential capacity for milk secretion of the pregnant cow as compared with the farrow cow. The vertically and horizontally shaded portions together represent the prepartum rate of inhibition of milk secretion due to pregnancy.

It is assumed that the source of the inhibitor is removed with the birth of the fetus so that the supply to the maternal circulation is cut off abruptly at parturition. A certain amount of inhibitor is at that time left in the maternal body and gradually disappears. The stippled portions of the ordinates represent the postpartum rate of inhibition of milk secretion due to this residue.

It is evident that if this residue is destroyed or eliminated at a rate proportional to its concentration the postpartum rate of inhibition may be represented by be^{-kt}, which subtracted from equation (1) would lead precisely to equation (5). There is some experimental evidence of the presence of an inhibitor to milk secretion in the maternal circulation both preceding and following parturition, and the considerations presented diagrammatically in Fig. 4 offer an alternative interpretation of equation (5).

Teleologically, one might say that gestation provides a mechanism which insures the preparation of the mammary gland for the secretion of milk for the postnatal nourishment of the young; which inhibits the secretion, almost or quite entirely, preceding parturition; and which by the gradual removal of the inhibitor following parturition provides for some time an increasing milk supply to meet the increasing needs of the growing young.

SUMMARY.

The rate of milk secretion in farrow cows may be expressed as $\frac{dy}{dt} = ae^{-kt}$, in which y = yield and t = time from calving. Preg-

nancy causes a decrease in yield which may be expressed as $\dfrac{di}{dp} = be^{Kp}$, in which i = inhibition or decrease in yield and p = time from conception. The constant K appears to be the same for various groups but b is roughly proportional to a. The decrease in yield associated with pregnancy is interpreted as due to a hormone. The hormone hypothesis also affords an interpretation of the increasing rate of milk secretion which occurs for a short time following parturition.

THE DISSOCIATION CONSTANTS OF RACEMIC PROLINE AND CERTAIN RELATED COMPOUNDS.*

BY C. M. McCAY AND CARL L. A. SCHMIDT.

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Recent developments (1) in the field of protein chemistry apparently indicate that a relationship exists between the ability of the protein molecule to combine with acids and with bases and its content of certain amino acids. Such correlations are at best only qualitative, since they are necessarily limited by the inaccuracies in the estimation of the amino acids and by the lack of physicochemical data concerning the components of the protein molecule. Although considerable progress (2) has in recent years been made in the estimation of the dissociation constants of the amino acids, such data are still lacking for proline, oxyproline, hydroxyglutamic acid, serine, and possibly for other components of the protein molecule.

The difficulty in obtaining pure proline has probably been the chief obstacle in the way of determining its dissociation constants. The syntheses of Fischer and Zemplén (3) and of Sörensen (4) are not wholly satisfactory. Our own attempts in this field have been negative. The method which is usually used for the preparation of proline was devised by Fischer and Boehner (5). The product as ordinarily obtained is highly impure and may contain as much as 30 to 40 per cent of its nitrogen in the form of amino nitrogen. Even after repeated crystallization of the copper salt of proline the product obtained by Van Slyke (6) contained 5 per cent of amino nitrogen. Gortner and Sandstrom (7) have commented upon the fact that even after a considerable number of steps had been taken by them in the attempt to obtain pure proline, their product still contained about 10 per cent of the total nitrogen in the form of amino

nitrogen. They point out that possibly the pyrrolidine ring opens to some extent during the treatment with nitrous acid. Our success in obtaining a product which is practically free from amino nitrogen is due to recognition of the fact that the substance which usually contaminates proline is probably glycocoll, and it is this substance rather than proline which reacts with nitrous acid to give off nitrogen.

The following experiment was suggested to us by Dr. S. P. L. Sörensen. It would be expected that if the pyrrolidine ring opens on

TABLE I.

Temperature 21°C.   Barometer 756 mm.

Proline.		Blank.	
Shaking time.*	Nitrogen.	Shaking time.	Nitrogen.
min.	*cc.*	*min.*	*cc.*
5	0.29	5	0.11
5	0.25	5	0.11
5	0.25	40	0.14
15	0.30	205	0.24
15	0.30		
30	0.29		
30	0.32		
60	0.48		
60	0.44		
60	0.39		
60	0.42		
120	0.55		
120	0.61		

* Duplicate determinations were carried out in order to determine the experimental error.

treatment with nitrous acid, the reaction should be influenced by the time factor and there should be a gradual increase in the amount of nitrogen which is evolved. The results which are given in Table I do not indicate that this factor is one of any considerable magnitude. In the experiment 2 cc. portions of a solution which contained 0.29 per cent of proline nitrogen were shaken with nitrous acid in the usual manner for varying lengths of time and the nitrogen which was set free was determined. The increase in nitrogen at the end of the 2 hour period over that which was given off during the first 5 minutes

was less than 2 per cent of the proline nitrogen in solution. On the basis of the usual 5 minute period which is employed for the estimation of amino nitrogen, the nitrogen which is set free as the result of the breaking of the pyrrolidine ring during this time does not account for more than 0.1 per cent of the proline nitrogen. Estimation of the amino nitrogen was subsequently used as an index of the purity of our proline preparations.

The impure proline whose purification was attempted in the following experiments was prepared from gelatin in the usual manner by hydrolyzing with 50 per cent barium hydroxide and extracting the barium-free, dried amino acid mixture repeatedly with absolute alcohol. The alcohol was evaporated and the resulting residue was again extracted with absolute alcohol. The process was repeated until a product was obtained which readily dissolved in absolute alcohol. The attempts at further purification were carried out with this product.

1. In a recent study of the structure of the dipeptide glutathione, Quastel, Stewart, and Tunnicliffe (8) employed unsymmetrical trinitrotoluene to determine the position of the free amino group on the peptide. They note that unsymmetrical trinitrotoluene will react with amino groups but not with certain imino structures. Our experiments with trinitrotoluene were carried out with the hope that this agent might be used for the removal of the contaminating amino acid. The gamma isomer, 3, 4, 6-trinitrotoluene, was prepared by the method of Brady and Gibson (9). A specimen of proline which contained 37 per cent of amino nitrogen was dissolved in absolute alcohol, trinitrotoluene was added and the mixture was evaporated to dryness. The resulting brown, viscous mass was heated on the water bath with 20 per cent sulfuric acid and filtered hot. A brown precipitate resulted upon cooling. The ratio of amino nitrogen to the total nitrogen in the filtrate was approximately the same as at the beginning of the experiment. This method evidently is of no value as a means for the purification of proline.

2. Under proper conditions proline probably reacts with nitrous acid in the manner characteristic of secondary amines.

$$HNO_2 + R_2 \cdot NH \rightarrow R_2N - N = O + H_2O$$

To remove the nitro group which attaches itself to the imino nitrogen requires boiling with acid. This drastic treatment was found to lead to extensive destruction of proline, probably due to the action of nitric acid which is formed in the reaction. Treatment of proline with amyl nitrite and hydrochloric acid in the cold, or hot, was found to be ineffective in reducing the content of amino nitrogen. Experiments in which proline was treated with sodium nitrite and sulfuric acid in excess showed that although the content of amino nitrogen could be reduced to about 5 per cent of the total nitrogen present there was invariably a loss of about 15 per cent of proline. Further attempts at diazotizing resulted in little reduction of the amino nitrogen and a heavy loss of proline.

3. The use of phosphotungstic acid suggested itself as a possible agent for the purification of proline. Sörensen (4) as early as 1905 showed that proline phosphotungstate is quite insoluble in 5 per cent sulfuric acid. Gortner and Sandstrom's attempts (7) at purification of proline included treatment of the impure product with phosphotungstic acid followed by several crystallizations of the copper salt. These procedures did not yield a pure product. Our attempts at purification were carried out in the reverse order to that followed by Gortner and Sandstrom, since it was suspected that the contaminating amino acid, when present in sufficient concentration, is likewise precipitated by phosphotungstic acid. Levene and Beatty (10), as well as Drummond (11), have shown that under proper conditions glycocoll and certain amino acids other than the hexone bases are precipitated from solution by phosphotungstic acid. A preparation of proline which contained 42 per cent of amino nitrogen was converted into the copper salt and recrystallized six times. This process resulted in a reduction of the amino nitrogen content to 1.5 per cent. Continued recrystallization is inadvisable, due to considerable loss of proline. Qualitative experiments indicated that only a slight precipitate of proline phosphotungstate was formed, after the solution had been allowed to stand for 24 hours at 8°C., when the concentration of proline was less than 0.6 per cent while heavy precipitates were obtained with 2.4 per cent solutions of proline. The final purification of proline was accomplished by precipitation of the proline containing 1.5 per cent of amino nitrogen by phosphotungstic acid, recrystallization of the

precipitate from hot water, and subsequent decomposition of the precipitate by means of barium hydroxide and removal of barium with sulfuric acid. 2 cc. of the resulting proline solution, containing 0.2 per cent of proline nitrogen, when treated with nitrous acid for 5 minutes, yielded within the limits of error no amino nitrogen. This specimen of racemic proline was used for the estimation of its dissociation constants.

In our attempts to synthesize proline a number of closely related compounds were prepared. This afforded the opportunity of determining the influence of structure upon dissociation constants. Pyrrolidone-α-carboxylic acid was prepared from glutamic acid according to the method of Haitinger (12) by heating for ½ hour at 150–160°C.

X Pyrrole-α-carboxylic acid
O Pyrrolidone-α-carboxylic acid
△ Proline
▫ Tetrahydro pyromucic acid

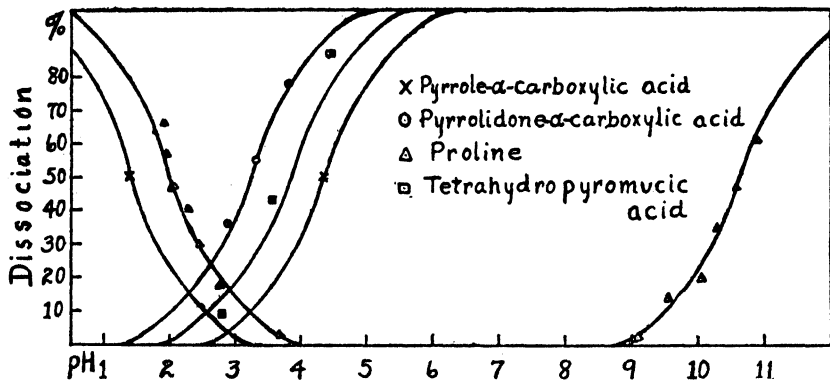FIG. 1.

The amide of the α-carboxylic acid of pyrrol was prepared from ammonium mucate according to the method of Schwanert (13) and subsequently converted into the free acid by hydrolysis with barium hydroxide; the barium was removed with sulfuric acid, followed by ether extraction. Tetrahydropyromucic acid was prepared according to the method of Adams and Voorhees (14). Although this is the best available method it yields a product which is not absolutely pure, but is sufficiently so to indicate the magnitude of its dissociation constant.

For the estimation of dissociation constants the well known methods which have been described by Michaelis (15) and by Clark (16) were followed. In brief, the method consists in determining the hydrogen

ion concentration in solutions of the ampholyte to which varying quantities of standard acid or alkali have been added. Estimation of the pH was made in the usual manner with the aid of the Clark cell, N/10 KCl-calomel electrode, saturated KCl bridge, and a Leeds and Northrup hydrogen ion potentiometer. The system was carefully checked with the aid of potassium phthalate mixtures and by titration of glycocoll. The calculations were made with the assistance of the tables of Schmidt and Hoagland (17). The usual correction for the water blank was made (18).

The titration curves are graphically shown in Fig. 1 and the values for the dissociation constants and isoelectric points are given in Table II. The results indicate that proline functions as a weak ampholyte. Comparison of the dissociation constants of the various

TABLE II.

Ampholyte.	K_a	K_b	Isoelectric point pH.
Racemic proline..........................	2.5×10^{-11}	1×10^{-12}	6.3
Pyrrole-α-carboxylic acid....................	4.2×10^{-5}	3×10^{-13}	2.9
Pyrrolidone-α-carboxylic acid...............	5.6×10^{-4}		
Tetrahydropyromucic acid.................	1.4×10^{-4}		

related compounds apparently indicates that the presence of oxygen as well as the unsaturated state of the ring both influence the magnitude of the acid dissociation constant. The titration curve of pyrrolidone-α-carboxylic acid gives no suggestion that the $-$COHN$-$ group plays any rôle in the neutralization of either acid or base. This is in agreement with inferences which may be drawn from the work of Eckweiler, Noyes, and Falk (19).

SUMMARY.

1. It has been experimentally shown that breaking of the pyrrolidine ring of proline by nitrous acid is not a factor of sufficient magnitude to account for the amino nitrogen which is usually found in proline preparations.

2. A method for the preparation of racemic proline is described. The product was found to be free from amino nitrogen.

3. The dissociation constants of racemic proline and of certain structurally related compounds were determined.

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A CONCENTRATION GRADIENT IN CORN STALKS.

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In a recent paper, Evelyn I. Fernald[1] has shown that the osmotic concentration of the sap of different portions of stems is correlated with the tendency of buds to develop on those portions. She has found by freezing point measurements on the expressed sap that in actively growing stems of privet, chestnut, *Philadelphus*, and *Bryophyllum* there is a concentration gradient, the highest concentration being in the apical portions, the lowest in the basal portions. In connection with these findings of Fernald on dicotyledonous stems, some measurements of the specific gravity of juice expressed from successive internodes of the corn stalk may be of interest.

The plants used in this study were all vigorous, 8 to 11 feet high, with large but immature ears. The juice from single internodes was expressed for the specific gravity measurements, except in the upper portions of the stalks where it was always necessary to take two or more adjacent internodes as one sample in order to obtain enough juice. Occasionally, also, the two basal internodes were expressed together, as indicated by brackets in the table.

After removal of the tough cortex, the tissue was thoroughly crushed in a food chopper. The juice was squeezed from the pulp by hand through a muslin cloth, and filtered until clear through a folded filter paper. Precautions against evaporation were observed throughout.

The specific gravity of the juice was determined by means of a small pycnometer in the form of a glass capsule with capillary intake and outlet tubes. A second weighing of the pycnometer filled with juice refiltered through the same paper constituted a check on each measure-

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[1] Fernald, E. I., *Am. J. Bot.*, 1925, xii, 287.

ment. The two weighings practically always agreed within 0.0002 gm. The specific gravity of each sample of juice given in Table I is the ratio of the weight of the sample to the weight of an equal volume of distilled water at approximately the same temperature as the juice.

To compensate for any cumulative changes in the cut stalks on standing, the order of expression and measurement of the juice from

TABLE I.

The Concentration Gradient in the Juice of Corn Stalks as Shown by Specific Gravity Measurements.

Internode (numbered from base of stalk).	Individual plant number.					
	1	2	3	4	5	6
1	1.0347	}1.0308	}1.0325	1.0255	}1.0452	1.0346
2						
3	1.0340	1.0304	1.0328	1.0255	1.0458	1.0376
4		1.0323	1.0331	1.0265	1.0486	
5	1.0404	1.0353	1.0358	1.0312	1.0519	1.0382
6	1.0418	1.0371	1.0376	1.0334	1.0522	
7	1.0433	1.0385	1.0397	1.0355	1.0530	1.0395
8	1.0448	1.0394	1.0400	1.0365	1.0534	1.0398
9	1.0452	1.0401	1.0424	}1.0383	}1.0542	1.0404
10*	1.0463	}1.0416	1.0458			}1.0413
11*	}1.0479		}1.0473	}1.0398	}1.0561	
12						
13	}1.0497	}1.0428	}1.0496			}1.0427
14						
15						
16						

* Ears borne on tenth and eleventh nodes of all these stalks.

the different internodes was varied in the different experiments. In some, the measurements proceeded from the lowest internode upwards to the top of the plant; in others, from the top downwards; and in still others, from the middle of the stalk alternately upwards and downwards. But no effect of the sequence of the determinations was discernible.

In Table I are given the data for six representative stalks, on each of which eight or more measurements were made. Similar gradients

were found in all of the thirty-eight stalks examined. The plants were cut on different days, so that the differences in the absolute values characterizing the individual stalks are probably due to differences in soil moisture and in the other environmental factors which have been shown to affect sap concentration.

It is interesting to note that in many stalks the specific gravity values for the two or three lowermost internodes do not conform to the gradient which is so well defined in every case above the third internode. With the exception of this frequent irregularity at the base of the stalks, the data are in accordance with the cryoscopic measurements reported by Fernald,[1] and with those on the gradient in trees, reported by Dixon and Atkins,[2] Chandler,[3] and Harris, Gortner, and Lawrence.[4]

[2] Dixon, H. H., and Atkins, W. R. G., *Scient. Proc. Roy. Dub. Soc.*, 1916, xv, 51.

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THE ISOELECTRIC ZONE OF TYPHOID AGGLUTININ.

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The isoelectric range of typhoid agglutinin, as reported by Michaelis and Davidsohn (1), is from pH 5.3 to 6.0 and as reported by von Szent-Györgyi (2) from pH 5.4 to 5.7. These authors used for their electrophoresis experiments typhoid-agglutinating serum diluted from 1:12 to 1:14 with buffer solutions.

It has been shown by Northrop and De Kruif (3) that the addition of egg albumin to suspensions of bacillus of rabbit septicemia has the effect of bringing the isoelectric point of the bacteria to approximately that of the albumin. These authors doubt that pH 5.3 to 6.0 is the isoelectric zone of typhoid immune body, since the presence of proteins has such marked effect on the position of the isoelectric point. von Szent-Györgyi made the observation that the isoelectric zone of immune body corresponds to that of serum globulin, about pH 5.4 as determined by Rona and Michaelis (4). The question then arises as to whether the zone of pH 5.3 to 6.0 is the isoelectric zone of the antibody itself, or of the serum globulin with which the antibody is known to be closely associated and which was present in comparatively large amounts in the diluted serum with which Michaelis and Davidsohn and von Szent-Györgyi worked.

Since the partially purified typhoid agglutinin solution produced by the dissociation of the immune body from sensitized organisms in our previous study (5) had but traces of serum protein in it (total nitrogen content not over 5 mg. per 100 cc.) it was thought worth while to make a determination of the isoelectric zone of the agglutinin by electrophoresis of this material.

Technique.

The apparatus used was essentially that described by Sherman, Thomas, and Caldwell (6) in their work on the isoelectric point of

malt amylase, with a few modifications. The U-tube electrophoresis apparatus of Michaelis as modified by these authors was adapted to drawing off samples from different parts of the tube by placing stop-cocks at the two lower corners of the U, and at a point half way between the large stop-cocks and the tops of the side arms. These additional stop-cocks make it possible to draw off samples from different parts of the tube without undue mixing. (See Fig. 1.)

FIG. 1. Electrophoresis tube arranged for removal of samples at different levels.

.The solution of antibody dissociated by N/200 NaOH from typhoid bacilli previously sensitized with antityphoid horse serum was first brought to approximate neutrality and was mixed with an equal volume of Sörensen's citrate buffer mixture of appropriate pH and weighted with 4 per cent of c. p. sucrose. In most of these mixtures a flocculent precipitate gradually settled out; but as it did not contain any agglutinin and did not interfere with the transport of agglutinin it

was disregarded.   Enough of this mixture was placed in the U-tube
to fill the bend and the bore of the large stop-cocks.   The apparatus
was then leveled off and the large stop-cocks closed.   The side arms
above the stop-cocks were rinsed out and then filled with the same
citrate buffer (here diluted 1:1 with distilled water) as was used to
mix with the antibody solution.   The leveling bulbs and Fales elec-
trode vessels were also filled with this diluted buffer and leveled off
by means of the leveling stop-cock (a) in the figure.   The electrodes
(copper in copper sulfate) were then inserted and the current (110
volts D.C.) turned on with the stop-cocks of the electrode vessels (b)
and the leveling stop-cock open.   The leveling stop-cock was then
tightly closed and the large stop-cocks in the side arms carefully
opened.   Tests showed that there was no diffusion of copper from
the electrodes through the leveling bulbs and into the side arms of the
U-tube in 24 hours when the stop-cocks of the electrode vessels were
left fully open.   For this reason they were not closed during the elec-
trophoresis, as was done by Sherman, Thomas, and Caldwell.   Under
these conditions the current passed was slightly over 1 milliampere.
The whole of the U-tube up to within about 1/2 inch of the top of the
side arms was immersed in a bath of running water which for all our
runs did not vary in temperature beyond the limits of 18–22°C.

After the material had been subjected to the influence of the current
for 24 hours the large stop-cocks in the side arms were carefully closed
and the current discontinued.   The contents of the U-tube were then
removed in seven portions in the following order (see Fig. 1): Portion
1 from upper half of cathode side arm and Portion 7 from upper half
of anode side arm by opening the small stop-cocks (c) half way up
the side arms.   Portions 2 (cathode side) and 6 (anode side) were
then pipetted out from the remainder of the side arms above the large
stop-cocks.   Portions 5 (anode side below large stop-cock), 4 (bottom),
and 3 (cathode side below large stop-cock) were then removed suc-
cessively by so tilting the U-tube as to bring the small stop-cocks
in the lower corners to a horizontal position in the proper order.
Each of these portions was tested separately for the presence of ty-
phoid agglutinin by the technique described in previous papers (5).
As a control a portion of the same solution as that which went into
the bend of the U-tube was placed in a test-tube and hung in

the water bath during the run.   This was titrated for agglutinin at the same time as the electrophoresed material.   Measurements of pH were made on the combined material from the bottom of the U-tube after electrophoresis.   The instrument used was a Leeds and Northrup type K potentiometer and a bubbling electrode

TABLE I.

pH	Titre of the control.	Titre of the + tube.	Titre of the − tube.
8.2	+320	+160	0
7.7	+640	+160	0
4.98	+320	+ 80	20(?)
4.95	+640	+ 40	0
4.94	+320	+ 80	0
4.87	+320	20(?)	0
4.75	+320	20(?)	0
4.70	+320	20(?)	0
4.64	+320	+ 20	0
4.51	+320	0	+ 20
4.50	+320	0	20(?)
4.48	+320	0	20(?)
4.40	+320	0	0
4.37	+320	20(?)	+ 20
4.25	+320	0	+ 40, 80(?)
3.95	+160	0	+ 80
3.94	+320	0	+160
3.6	+640	0	+320
3.4	+320	0	+160
2.5	+640	0	+320

+ + = complete, + = partial, (?) = weak agglutination at the dilution indicated.

RESULTS.

A summary of results obtained and of the agglutinations in the critical material in the inch just above the U-tube is given in Table I. It will be seen that the direction of transport in the electrical field is definitely dependent upon the hydrogen ion concentration of the solution.   These results differ from those previously reported for typhoid agglutinin in that the isoelectric zone of the antibody is not from pH 5.4 to 6.0 but from about 4.4 to 4.9 with a turning point somewhere between pH 4.5 and 4.65.

A sample of serum diluted 1:14 with buffer solution was then electrophoresed at pH 4.85. At this point, according to Michaelis and Davidsohn, the agglutinin should be on the acid side of its isoelectric zone and should travel toward the negative pole in an electrical field. It will be seen from Table II that this is not the case, but that the agglutinin traveled to the positive pole, showing that it was on the alkaline side of its isoelectric zone, and was negatively charged.

TABLE II.

Electrophoresis of Typhoid-Agglutinating Serum at pH 4.85.

One part serum to fourteen parts buffer solution. 110 volts D.C. for 36 hours. pH of solution after electrophoresis was 4.85.

	Dilution.								
	20	40	80	160	320	640	1280	2560	5120
+ side arm....................	++	++	++	++	++	++	+	0	0
Control......................	++	++	++	++	++	++	++	+	0
− side arm...................	0	0	0	0	0	0	0	0	0

The electrophoresis of typhoid agglutinin in serum was, therefore, further investigated with the following results (Table III).

TABLE III.

pH	Titre of control.	Titre of + side.	Titre of − side.
5.44	++2560	+640	+ 40
5.22	++2560	++160	0
		320(?)	
4.85	+2560	+640	+ 20
4.81	+2560	+640	+ 20
4.58	++1280	+ 80	++ 40
4.43	+5120	+ 80	+ 160
4.28	+5120	+ 40	+ 320
3.84	+5120	+ 80	+1280
3.81	+2560	0	+ 640
3.35	+2560	0	+ 640

It will be seen from this summary that the agglutinin in the serum behaves in the same manner under the influence of the electric current as the agglutinin in the serum-free solution. There is a turning point between pH 4.43 and 4.58.

On account of the difference between our figures and those of previous workers, nine of our solutions were taken to the chemical department of Columbia University where the pH was determined by Mr. Foster through the kindness of Professor Arthur W. Thomas. The results were entirely consistent with ours.

In several controls in which the apparatus was set up as usual but no current was passed, there was no transport of agglutinin in either direction.

The specificity of the agglutination was controlled by testing the purified agglutinin solution on *B. paratyphosus*, A and B, *B. dysenteriæ*, Flexner and Shiga, and *B. coli*—all with negative results. The antityphoid horse serum used was supplied by the New York Department of Health through the kindness of Dr. W. H. Park and Dr. Charles Krumwiede, to whom we are greatly indebted.

The only explanation we can offer for the different results is that Michaelis and Davidsohn worked with immune rabbit serum, and that von Szent-Györgyi, while he worked as we did with horse serum, made his hydrogen ion estimations colorimetrically.

The finding, if verified by others, should be of value in further attempts to isolate antibodies.

SUMMARY.

The isoelectric point of typhoid agglutinin lies between pH 4.4 and pH 4.6.

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THE COMBINATION OF SALTS AND PROTEINS.

II. A Method for the Determination of the Concentration of Combined Ions from Membrane Potential Measurements.

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The exact determination of the combination of proteins with the ions of electrolytes has been limited practically to those cases in which concentration cells can be used. Bugarszky and Liebermann[1] found by this method that the hydrogen ion is very largely combined and chloride to a much less extent. These measurements have been repeated and confirmed by a number of workers, and extended by Pauli and Matula[2] to Ag^+, which was found to be also quite largely combined. Pauli and Samec[3] also found that the solubility of slightly soluble salts was increased by proteins, and this furnishes an additional method for determining the amount of combined ions. Both methods, however, are limited to a very small number of ions and only in rare cases, such as HCl or $ZnCl_2$, is it possible to determine the combination of both ions. It was pointed out in the preceding paper[4] that the Donnan equilibrium furnishes a general method which can be used for any ion, provided the distribution of one ion and the analytical composition of the solutions be known. It was shown that using gelatin particles and determining the theoretical ion ratio from Cl^- electrode potentials, the concentration of Zn ions combined with the gelatin could be calculated, and agreed quite well with the values determined directly by concentration cell measurements. This method is also limited since it can only be used with gelatin and under certain conditions, since in alkaline solutions the chloride electrodes

[1] Bugarszky, S., and Liebermann, L., *Arch. ges. Physiol.*, 1898, lxxii, 51.

[2] Pauli, W., and Matula, J., *Biochem. Z.*, 1917, lxxx, 187.

[3] Pauli, W., and Samec, M., *Biochem. Z.*, 1909, xvii, 235.

[4] Northrop, J. H., and Kunitz, M., *J. Gen. Physiol.*, 1924–25, vii, 25.

cannot be used. It is evident from Donnan's theory that the desired potential is also given by the membrane potential, so that if this be accurately measured the ratios of the activities of all the ions inside and outside of the membrane can be calculated. Loeb[5] showed, by comparing the membrane potential with the hydrogen and chloride electrode potential, that the theory was completely borne out by experiment. If, therefore, a protein solution in a membrane be allowed to come to equilibrium with an electrolyte solution, and the membrane potential and ion concentrations determined, the effect of the protein on the activity of the various ions can be calculated. This furnishes a general method which can be used for any ion.

Theoretically the procedure is very simple, but experimentally there are a number of difficulties and several important sources of error. It is essential that the system be at equilibrium and also that no diffusion potentials enter at the various liquid junctions. It is impractical to measure the potential while the system is in osmotic equilibrium and it is necessary to show that the potential is not affected by removing the pressure. Agreement between electrode and membrane potential may be used as a test for all these errors, since the P. D. will agree only when these errors are eliminated. It was found possible, after considerable difficulty, to regulate conditions so that the membrane potential agreed closely with the electrode potentials, and the combined ions calculated from this membrane potential agreed with the results obtained from concentration cells.

Experimental Procedure.

The experiments were carried out with 10 per cent gelatin solution, at 37°C., using collodion membranes.

Preparation of the Membranes.—In order to avoid stretching of the membrane and the passage of small amounts of protein through it, it was found necessary to standardize conditions during the preparation. The following procedure was found to produce membranes which stretch very slightly if at all during the experiment and which were practically impermeable to gelatin at 37°C. They were used only once, since after the experiment they become very impermeable even to electrolytes.

7.5 cc. of Merck's U.S.P. collodion (4 per cent dry weight) were placed in a 175

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[5] Loeb, J., Proteins and the theory of colloidal behavior, New York and London, 1922.

× 20 mm. test-tube and the tube rotated mechanically in a nearly horizontal position at about 10 rotations per minute for 5 minutes, care being taken to prevent the collodion from running out over the lip of the tube. Air, under 1 cm. Hg pressure, was then blown from two 1.5 mm. tubes inserted so as to reach, one about half way, and the second nearly to the bottom of the test-tube. This was continued while the tube rotated for about 6 minutes longer depending on atmospheric conditions. It was then put in water and the membrane removed. The
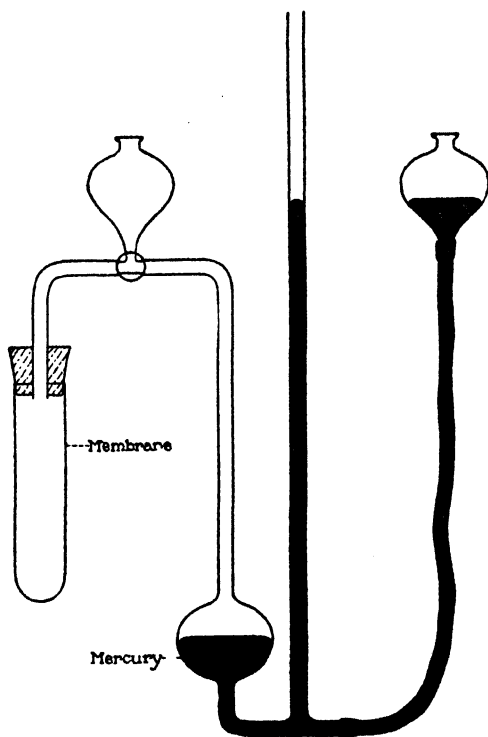
FIG. 1. Apparatus for testing membranes.

membrane was then attached to a rubber stopper by means of rubber bands, and filled with water. 20 cm. Hg pressure was then applied, and the membrane carefully examined for leaks. This pressure causes considerable stretching, so that no further stretching occurs during the experiment. At the same time the rate of flow of the water through the membrane was measured. This serves as a measure of the permeability, and the time of drying was so regulated as to keep this constant. The apparatus shown in Fig. 1 was found very convenient for this purpose.

Preparation of the Gelatin.—Powdered gelatin was washed in dilute alkali, then in dilute acetic acid, and finally at pH 4.7 with ice water, until the gelatin reached a specific conductivity of less than 5×10^{-6} reciprocal ohms. The resulting swollen particles were then melted. Such preparations usually contained about 17 gm. dry weight of gelatin per 100 cc.

Assembling the Apparatus.—Since the final calculation requires an accurate figure for the concentration of electrolyte, both in the gelatin and in the surrounding liquid, it is necessary to know the water content of the solution with considerable accuracy. This can best be done by weight. The concentrations, therefore, in this paper are all expressed as molal; *i.e.*, the number of mols of solute per 1,000
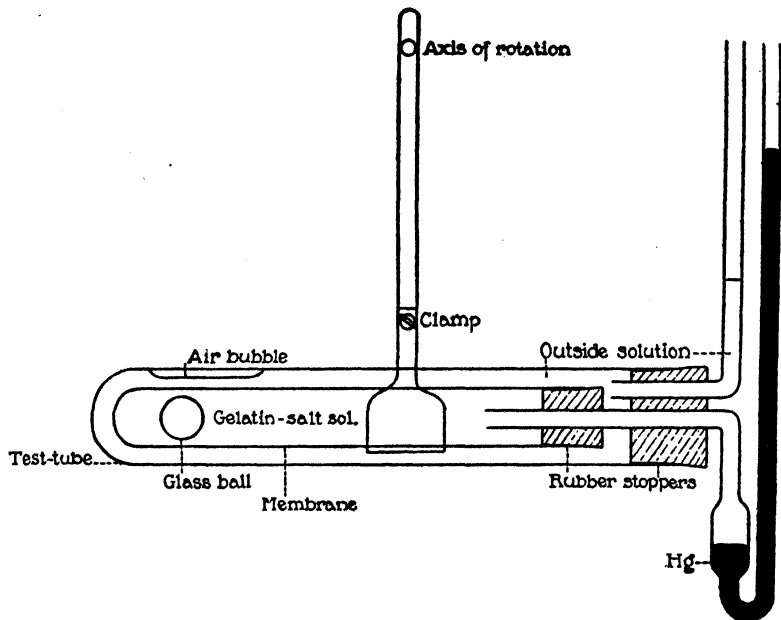
FIG. 2. Osmometer with stirring device.

gm. of water. This obviates also the uncertain correction for the volume occupied by the gelatin which occurs when volume concentrations are used. It was found necessary, in order to shorten the time required for equilibrium, to stir both inside and outside solutions. This was done by placing a large glass ball in the membrane and rocking the entire system, as shown in Fig. 2. The time is further shortened by starting the experiment with equal molalities of electrolyte inside and outside. Control experiments showed that the same value was reached if all the electrolyte was placed outside at first, but that a slightly longer time was required under such conditions. A gelatin solution is therefore prepared so as to contain 10 gm. of

gelatin per 90 gm. of water, and the desired amount of electrolyte. The membrane with its attached manometer and stopper (Fig. 2) is then weighed, filled with the gelatin solution, and again weighed. This gives the total weight of the inside solution and therefore the weight of gelatin. Similarly about 60 cc. of the same molal concentration of electrolyte is measured into a 100 cc. test-tube, and the membrane inserted and held in place by a rubber stopper. The tube is clamped to a rocker arm in the water bath, as shown in Fig. 2, and rocked for 48 hours. At the end of this time, the osmotic pressure is measured with a cathetometer, the manometer tube removed from the membrane, and a curved tube put in its place.
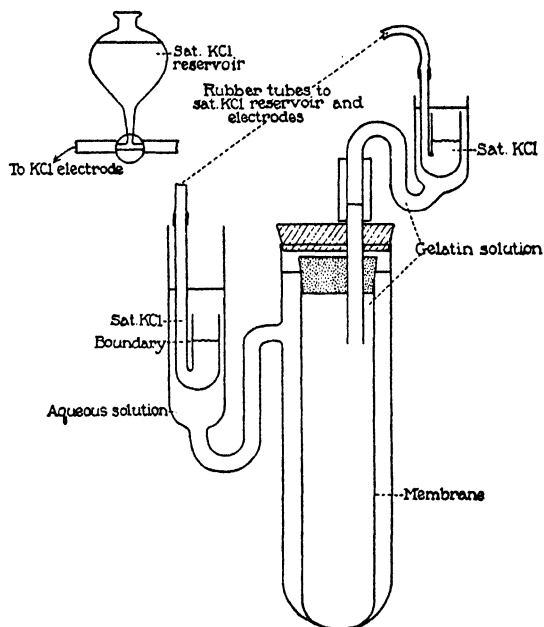
FIG. 3. Membrane arranged for potential measurement.

The outside solution is then poured into a tube with a side arm as shown in Fig. 3, and the membrane inserted in this tube. This tube is shorter than the original outside tube, so that the membrane is pressed against the bottom and sufficient of the inside liquid forced out to fill the bent tube. The arrangement is shown in Fig. 3.

The liquid in the two cups is now connected to two saturated KCl calomel electrodes by means of the bent tubes shown in the figure. In order to obtain constant and reproducible potential measurements it was found necessary to use considerable care in the establishment of the saturated KCl solution–liquid junction. The bent tubes must be quite wide (8 mm.) and are filled with saturated

KCl before inserting in the liquid. They are then washed, and the KCl is removed from about half the depth with a pipette. Liquid from the appropriate solution is then added from a dropper till the tubes are again full, and they are then inserted into the corresponding solution. The junction is then stirred. In this way a wide liquid junction is formed without any sharp boundary, and the potential reading is very constant and reproducible. If narrow tubes are used or if a sharp KCl boundary is formed, the potential drifts and is sensitive to stirring at the boundaries. A type K Leeds and Northrup potentiometer was used for the measurements with a type 2420a galvanometer whose sensitivity had been greatly increased by focusing the image on a scale about 6 m. distant instead of the usual 15 cm. The E.M.F. measured in this way was found to be constant and reproducible to about ±0.1 millivolt.

The Zn^{++} and Cl^- concentration cell measurements and electrode potentials between the inside and outside solutions were made by means of the electrode vessels shown in Fig. 4. The electrode vessels were filled with the appropriate solution and suspended in the water bath over the edge of a cup containing the
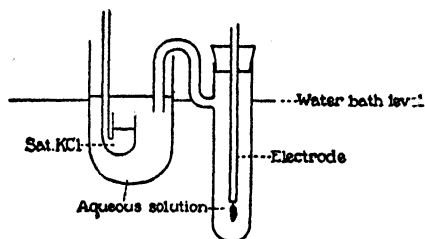
FIG. 4. Arrangement of electrodes for concentration cell measurements.

same solution. Connection with the calomel electrodes was then made in the same way as for the membrane potentials. In using the Zn electrodes it is necessary in order to obtain constant readings to completely fill the electrode vessel and in the case of aqueous solution to slightly acidify the solution.

Owing to the danger of contamination from KCl the analytical data were determined from a separate experiment in which the P.D. was not measured. For this purpose the membrane and its contents were removed and weighed (in order to determine the final concentration of gelatin), and a weighed sample of both inside and outside solution analyzed gravimetrically for Cl. The gelatin solution, after the addition of $AgNO_3$ and 1/3 of its volume of strong nitric acid, is boiled in order to effect complete precipitation. Since in these experiments only pure chlorides were used, the cation concentration can be calculated from the Cl determination. In the case of $ZnCl_2$ a small amount of HCl was added to bring the solution to pH 4.7. The amount of Cl added in this way was corrected for in determining the Zn. From the various weights and the Cl analysis it is therefore possible to calculate the molality of both ions in the outside solution and in the gelatin.

Calculations of the Combined Ions from the Membrane Potential and the Analytical Results.

According to Donnan[6] the membrane potential at equilibrium is equal to $\frac{RT}{F} \, ln \, \frac{\alpha_o}{\alpha_i}$, where α_o is the activity of any diffusible ion outside the membrane, and α_1 is the activity of the same ion inside. Also, $\alpha_o = \gamma_o M_o$ and $\alpha_i = \gamma_i M_i$, where γ = the activity coefficient and M is the molality of the uncombined ion. A change in α may evidently be due, therefore, either to a change in the activity coefficient γ or to a change in the molality, and at present there seems no way to distinguish with certainty between these alternatives.

According to Lewis and Randall,[7] however, the value of the activity coefficient of any ion in a mixture of strong electrolytes depends only on the "ionic strength" of the solution. A change in the activity due to a change in the activity coefficient should therefore affect the activity of all ions. In the case of proteins, however, this is not true, the activity of some ions (as H^+) being greatly affected by the addition of the protein, while others (as Cl) are only slightly affected. It will be assumed, therefore, in this paper that any change in the activity of an ion on the addition of a protein is due to a change in the concentration of the ion and not to any effect on the activity coefficient. Or, in other words, it is assumed that the protein does not change the "ionic strength" of the solution. In all the experiments reported in this paper the total salt concentration on the two sides of the membrane is very nearly the same, so that γ_o may be considered equal to γ_i, and the formula may therefore be written

$$\text{E.M.F.} = \frac{RT}{F} \, ln \, \frac{M_o}{M_i}.$$

(This, however, is not the case with HCl, since here the total acid concentration inside may be much greater than that outside, and it is necessary to use different activity coefficients; *cf.* Cohn and Berggren.[8])

[6] Donnan, F. G., *Z. Elektrochem.*, 1911, xvii, 572; *Chem. Rev.*, 1924–25, i, 73.

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Let
$$r = \frac{M_o}{M_i},$$

then
$$\ln r = \frac{\text{E.M.F.}}{\dfrac{RT}{F}}$$

or
$$\log r = 0.4343 \frac{\text{E.M.F.}}{\dfrac{RT}{F}}. \quad [9]$$

The ion activity ratios may therefore be calculated directly from the membrane potentials. The actual concentration of the combined ions, however, cannot be calculated without the analytical data. If the ratio of the activities is known from the membrane potential as shown above, and also the total concentration of the ions on both sides of the membrane, then the concentration of combined ions, M_c, is given by the equation

$$M_c = M_i - \frac{\gamma_o M_o}{\gamma_i r}$$

or, if
$$\gamma_o = \gamma_i$$

$$M_c = M_i - \frac{M_o}{r}$$

where M_i is the total molal concentration of the ion inside and M_o is the total molal concentration of the ion outside the membrane.

[9] In using this formula it avoids confusion as to the sign of the potential, if it is remembered that all ions which have the same sign as the calomel electrode in the gelatin (referred to the outside calomel electrode), will have a greater concentration outside the membrane; while all oppositely charged ions will be more concentrated inside the membrane. All electrode potentials will therefore show (between the electrodes) the opposite sign to that of the membrane potential. This calculation is essentially the same as the calculation of combined ion concentration from concentration cells.

Comparison of Electrode and Membrane Potentials and of the Concentration of Combined Ions from Concentration Cell and Membrane Potential Measurements.

The membrane potentials and the potentials obtained with the various electrodes between the inside and outside solutions are given in Table I. The agreement between these figures shows that the system was in equilibrium and confirms Loeb's results that such a system conforms to Donnan's theory with considerable accuracy.

TABLE I.

Comparison of Electrode and Membrane Potentials.

Electrolyte.	ZnCl$_2$	KCl			HCl
Approximate total molality..............	0.10	0.05	0.25	1.0	0.06
Membrane P.D., millivolts,					
Hg HgCl sat. KCl | MeCl | membrane 　　　　　　 | H$_2$O |	1.14				
|MeCl|sat. KCl HgCl Hg............	1.24	−0.90	−0.40	−0.08	22.2
|gel |	1.50				
Zn electrode P.D., millivolts,					
Zn |ZnCl$_2$| sat. KCl| ZnCl$_2$| Zn...........	−1.50±0.02				
|H$_2$O | 　 | gel |					
Ag-AgCl P.D., millivolts,					
Ag AgCl|MeCl|sat. KCl|MeCl|AgCl Ag..	−1.12	0.74	0.33	0.05	
| H$_2$O | | gel |					
H$_2$ electrode P.D., millivolts,					
PtH$_2$|HCl|sat. KCl|HCl|H$_2$Pt...........					−23.0
|H$_2$O| | gel|					

A comparison of the combined ions from these measurements is shown in Table II. The agreement is again within a few per cent, except in the case of chloride in high concentration. In this case the figure from the membrane potential depends on a very small difference between two large numbers, so that an error of 1 per cent in analysis may amount to 50 per cent in the figure for combined chloride. All the ions show some combination although in the case of lithium the amount is close to the limit of accuracy of the measurement. It may also be noted that in no case are the amounts of anion and cation

combined equivalent. With $ZnCl_2$, for instance, less than 1 mol Cl is combined per mol of Zn. It is not possible to draw any quantitative conclusion from a comparison of the different ion values since, except in the case of H, they are undoubtedly not maximum values but would increase with increasing concentration of the electrolyte.

TABLE II.

Comparison of Values for Combined Ions by Different Methods.
10 Per Cent Gelatin.

Method.	Electrolyte.	Ion.	Total concentration of ion.	Concentration of combined ion.	Per cent total concentration combined.	Millimols combined per gm. gel.
Concentration cell (37°C.)...............	$ZnCl_2$	Zn	0.010	0.0063	63	0.063
" " (25°C.)...............	"	"	0.011	0.0068	62	0.068
Membrane P.D........................	"	"	0.0126	0.0066	52	0.066
Concentration cell (37°C.)...............	"	"	0.10	0.028	28	0.28
Membrane P.D........................	"	"	0.112	0 027	24	0.27
Concentration cell (37°C.)...............	LiCl	Cl	0.10	0.0068	6.8	0.068
Membrane P.D........................	"	"	0.1049	0.008	7.6	0.08
	"	"	0.106	0.0096	9.1	0.09
" "	"	Li	0.1049	0.0035	3.3	0.035
	"		0.106	0.0050	4.8	0.05
Concentration cell (33°C.*)...............	HCl	H				0.92
Membrane P.D. (4.67 per cent gel).......			0.06	0.0428	70	0.916
			0.06	0.0427		0.917

* Hitchcock, D. I., *J. Gen. Physiol.*, 1921-22, iv, 739.

SUMMARY.

A method is described for measuring membrane potentials of gelatin-salt solutions, and it is pointed out that such measurements, together with the analysis of the solutions, allow the calculation of the concentration of ions combined with the protein.

The values for the combined ions obtained in this way for $ZnCl_2$, KCl, LiCl, and HCl agree quite well with those obtained by direct concentration cell measurements.

ELECTROENDOSMOSIS THROUGH MAMMALIAN SEROUS MEMBRANES.

III. The Relation of Current Strength and Specific Resistance to Rate of Liquid Transport. Transport Rate with Serum.

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The present experiments concern the relation of the electric current strength and of the specific electrical resistance of the perfusing liquid to the rate of electroendosmotic flow across mammalian serous membranes. A direct proportionality is found between liquid and electric flow through these membranes, which are complex in structure and heterogeneous in composition. The variables Φ and I are thus connected in the complex case by the same relationship as in the case of simple membranes to which the classical electroendosmotic equation applies. Less simple relations are found when the membranes are bathed in buffers of varying specific resistance. Quantitative determinations are also reported with whole serum and the membranes of living and dead animals. The rate of electroendosmotic flow across dog and cat serosæ bathed in serum has been found to be 0.2 to 0.3 c.mm. per minute per milliampere toward the cathode.[1]

Relation of Current Strength to Rate of Flow.

The experimental set-up is shown in Fig. 1. The membranes were fastened by broad rubber bands over the mouth of the electrode vessel; the inside diameter

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[1] The ratio of current strength to liquid flow through any given membrane is independent of the area of membrane through which the current is passing. For if a given constant potential difference is maintained across the membrane, the current strength and the volume of liquid transported in unit time will both be proportional to the area of the membrane through which flow occurs. The dimension of area therefore cancels out and does not appear in the ratio of liquid to electric flow.
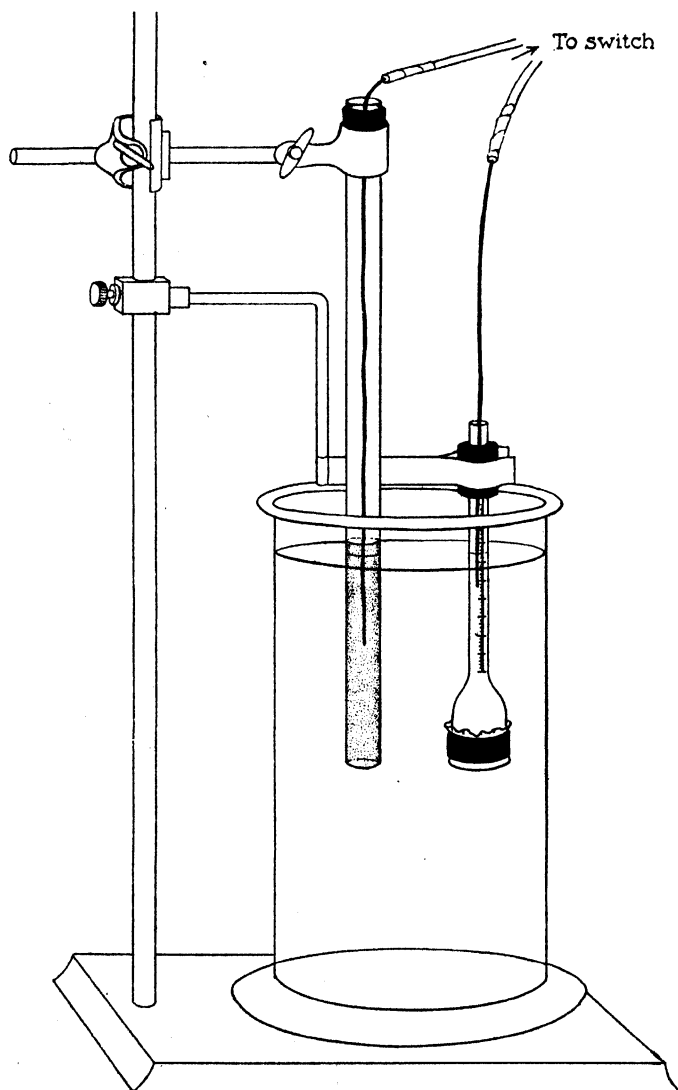
FIG. 1. Arrangement for study of electroendosmotic transport across animal pericardia.   See text.

of the mouth was 18 mm. The electrode vessel was filled and emptied with capillary pipettes; it dipped into a large vessel of buffer containing an agar electrode. Current was led into the buffer in the electrode vessel by a platinum wire. The buffer within and without the electrode vessel was the same.

The source of E.M.F. was connected to the two ends of a 666 ohm slide-wire rheostat. The experimental lines were led off, one from one end of the rheostat and the other from the rheostat slider. By moving the slider, therefore, the
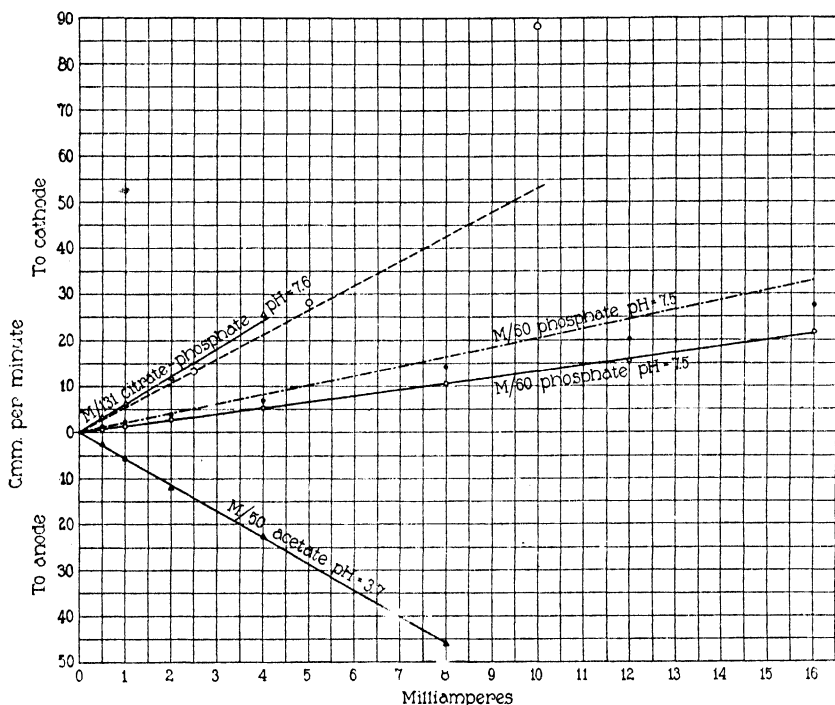
FIG. 2. The relation between volume of liquid transported in unit time (ordinates) and electric current strength (abscissæ) for several systems.

P.D. between the experimental lines could be varied continuously from zero to the total applied E.M.F. A pole-charging switch and a Weston standard milliammeter were included in the circuit. The experimental procedure finally adopted gave satisfactorily reproducible results with a minimum of error due to temperature changes, leakage, bulging of the membrane, polarization, etc.

The data obtained are presented in Fig. 2. The points fall with considerable accuracy along the solid straight lines, and these pass

through the origin; the proportionality of liquid and electric flow even with minimal current strengths is thus indicated.

The points do not fall satisfactorily along the broken lines. In the case of the dash line (M/131 citrate-phosphate data) the erratic position of the points above 4.0 milliamperes is almost certainly due to the effect upon the membranes of chemical changes about the platinum electrode. In these experiments the electrode was brought near the membrane in order to obtain the desired current strengths. Later the electrode was kept well up in the narrow vertical tube.

In the case of the broken line with M/60 phosphate mixture, a disturbing factor seems to have been that the membranes were applied very laxly over the mouth of the electrode vessel.

The slopes of the straight lines indicate the rate of liquid transport per milliampere characteristic for each system. These are:

Buffer mixture of Na_2HPO_4 and citric acid; molarity, M/131; pH 7.6. Solid line, composite of two experiments with lean cat pericardia. Rate of liquid transport, 6.00 c.mm. per milliampere per minute to cathode. Dash line, composite of three experiments with cat pericardia.

Buffer mixture of Na_2HPO_4 and KH_2PO_4; molarity, M/60; pH 7.5. Solid line, lean pericardium of male dog; runs continued throughout 6 working days. Rate of liquid transport, 1.33 c.mm. per milliampere per minute to cathode. Experimental site at end of experiment composed chiefly of bundles of collagen fibres in two more or less well defined lamellæ; elastin fibres among collagen bundles; basement membrane persistent only in places; mesothelium gone; in one region a zone of looser connective tissue containing fat cells, blood vessels, and a nerve. Fibre bundles considerably frayed out. Dot-dash line, composite of three experiments with dog pericardia; a few runs only with each membrane.

Buffer mixture of acetic acid and sodium acetate; molarity, M/50; pH 3.7. Lean pericardium of male dog; runs through 4½ working days. Rate of liquid transport, 5.76 c.mm. per milliampere per minute to anode. Experimental site of dense zones of collagen fibres with a few elastin fibres interspersed; in a part of section looser connective tissues containing fat cells and blood vessels. Basement membrane persistent in places only; mesothelium gone.

Relation of Specific Resistance of Buffer to Rate of Flow.

The buffers used were Sörensen's phosphate mixtures to which NaCl was added to give the desired conductivity. The stock KH_2PO_4 and Na_2HPO_4 solutions were mixed and diluted to give buffers of approximately 7.4 pH and M/60 concentration. Sodium chloride was added in amounts such as to give a series of M/60, M/50, M/40,

M/30, M/20, M/10, M/7, and M/6 total molar concentration. The pH was readjusted to about 7.4 by addition of dilute NaOH. The specific resistance of the buffers was determined at 21.6° ± 0.2°C. by the ordinary Kohlrausch method. The viscosity of the M/6 buffer was found to be only a little more than 2 per cent greater than that of distilled water. Viscosity measurements were thereafter
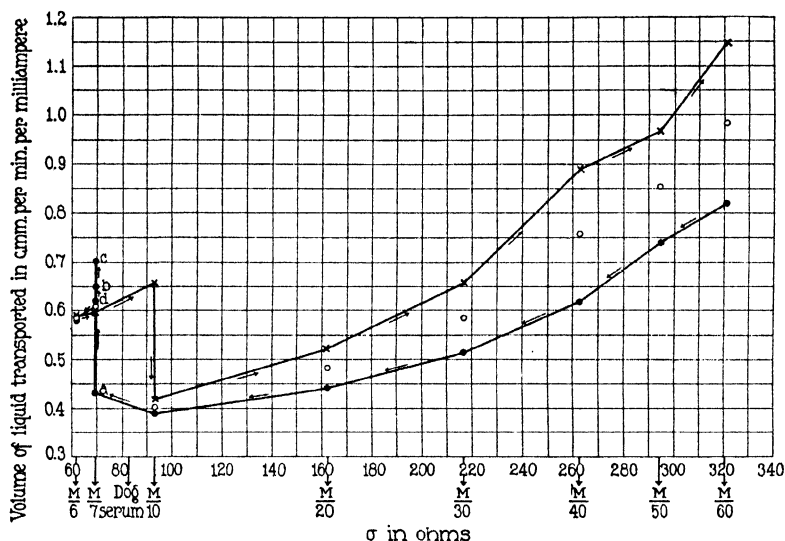
FIG. 3. Experiment 1. Relation of rate of electroendosmotic transport to specific resistance of buffers bathing membrane. Male dog pericardium. Current strength 15 milliamperes. pH of buffers 7.36 to 7.44. Arrows and letters indicate order in which buffers were used. Mean values for each buffer plotted as white circles.

discontinued, since fluctuations in room temperature and heating effects with passage of current undoubtedly caused variations in viscosity greater than this.

The experimental arrangement has already been described (Fig. 1). Eight preliminary runs with inner electrode alternately cathode and anode were routinely made with each buffer to impregnate the membrane. Three pairs of runs were then made with 15 milliamperes current. The mean rates of liquid transport for these last runs are plotted against the specific resistances of the several buffers in Figs. 3 and 4.

In a simple homogeneous membrane of constant structure and composition the rate of electroendosmotic transport per unit of current is proportional to the specific resistance (σ) of the solution in the membrane pores.   Were the present membranes of this nature (see, however,[2]) the plots of volume transported against specific resistance should be straight lines, or should deviate appreciably from straight lines only through changes in the electrokinetic P.D.   How far the
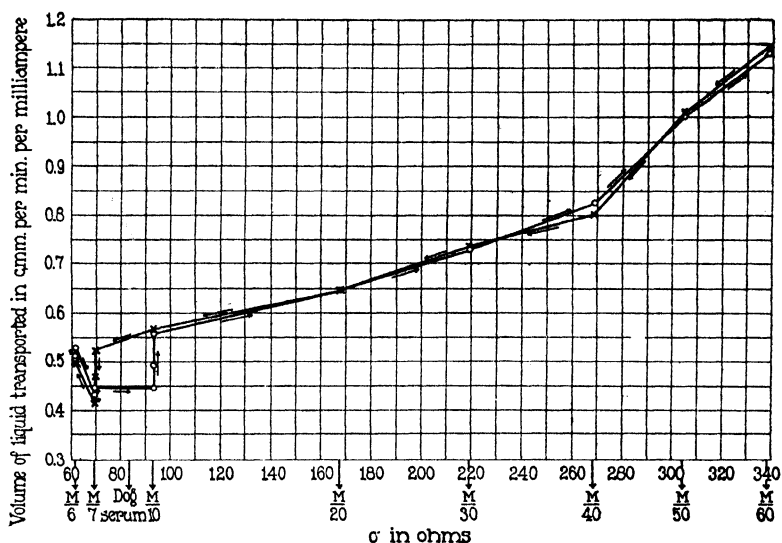
Fig. 4. Experiment 2.   Rate of transport and specific resistance of buffers. Female dog pericardium.   Current strength 15 milliamperes.   pH of buffers 7.37 to 7.41.   Arrows indicate order in which buffers were used. σ of M/7 and M/10 approximate only.

behavior of the present membranes differs from that of a simple inert membrane is shown by the discontinuity of the lines to the left-hand side of Figs. 3 and 4.

Two characteristics of the experimental curves are especially to be noted:

First, that the slope of the lines from σ = 93 ohms increases with increasing values of σ.   An increase in electrokinetic P.D. between solid and solution with decreasing salt content is a general phenomenon

[2] Mudd, S., *J. Gen. Physiol.*, 1924–25, vii, 389.

in the range of concentrations here used[3] and undoubtedly contributed to the upward trend of the lines with increasing values of σ. However, in consideration of what follows this explanation seems incomplete.

Second, the remarkable discontinuity of the curves in the region in which the conductivities and osmotic pressures of the buffers are close to those of the blood. Each of the points plotted is the mean value of six 6 minute runs. The buffer in the electrode vessel was changed after each run and other delays were incurred. The several points for any one buffer are therefore separated by intervals of from 1

TABLE I.

Experiment No.	Membrane.	State of animal.	Current strength.	Buffer.				σ of buffer.	Rate of transport per milliampere.	σ of serum of experimental animal.
			milli-amperes					*ohms*	*c.mm. per min.*	*ohms*
3	Dog mesentery.	Living.	25	(Phosphates $+$ NaCl)			M/6.	61.83	0.36	83.54
	Dog mesentery.	"	25	"	"	"	M/7.	—	0.29	83.54
	Dog mesentery.	"	25	"	"	"	M/10.	—	0.39	83.54
4	Cat pericardium.	Dead.	15	"	"	"	M/6.	61.83	0.57	83.27
	Cat pericardium.	"	15	"	"	"	M/7.	69.68	0.49	83.27
	Cat pericardium.	"	15	"	"	"	M/10.	93.09	0.57	83.27

to several hours, and an overnight stay in buffer in the ice box usually intervened between the runs with the different buffers of $\sigma = 93$ ohms or less. The "hysteresis loops" at the left-hand side of Figs. 3 and 4 thus represent slow changes with time as well as with changing buffers in the rate of electroendosmotic transport across these pericardial membranes. These changes appear to have been completely

[3] Northrop, J. H., and De Kruif, P. H., *J. Gen. Physiol.*, 1921–22, iv, 639, 655. Northrop, J. H., and Freund, J., *J. Gen. Physiol.*, 1923–24, vi, 603. Loeb, J., *J. Gen. Physiol.*, 1922–23, v, 109, 395, 479; 1923–24, vi, 215.

reversible in Fig. 4, only partially so in Fig. 3. On what alterations in structure and composition within the membrane they depended can only be guessed. The equilibrium between membrane and environing medium was evidently very sensitive when the osmotic and electric conditions of the medium roughly approximated those of the blood.

The other two experiments affording data on rate of transport with the M/6, M/7, and M/10 phosphate and NaCl buffer mixtures show minimum values with M/7. See Table I.

Throughout Experiments 1, 2, and 4, and only these afford data on this point, the mean rate of transport to the cathode was greater with inner than with outer electrode cathode when buffers M/6 to M/20 were used, and the rate of transport to the cathode was greater with outer than with inner electrode cathode when buffers M/30 to M/60 were used. The differences were great at the extremes of the buffer series and gradually diminished to the transition point between M/20 and M/30. With M/60, for instance, the mean rise of the meniscus toward the inner cathode was to the fall of the meniscus toward the outer cathode as 1 is to 1.6; with M/6, mean rise to inner cathode: mean fall to outer cathode:: 2.1:1. These effects no doubt indicate that the membrane was polarized during the passage of current and that the polarization was somehow correlated with the conductivities of the perfusing buffers and with the arrangement of the membrane with respect to the inner and outer fluids.

Bethe and Toropoff[4] have demonstrated the polarization of diaphragms during passage of current. Reversal with acid of the direction of electroendosmotic flow causes also reversal of the direction of polarization. The relative disturbances in ion concentrations at the membrane surfaces are diminished by increasing salt concentrations in the perfusing solutions.

The Transport Rate with Serum.

· The apparatus used for serum experiments (Fig. 5) was slightly modified from suggestions made by Dr. M. Kunitz. A Zn-ZnSO$_4$ electrode connects through an L-way stop-cock with a salt bridge filled with 0.8 per cent NaCl solution. A second L-way stop-cock near the other end of the bridge facilitates control of

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[4] Bethe, A., and Toropoff, T., *Z. physik. Chem.*, 1914, lxxxviii, 686; 1914–15, lxxxix, 597.

the several solutions.[5]   The open-end vertical tubes are used for washing and
filling.   The rest of the arrangement is as already described.[2]   Readings of the
meniscus are made with the circuit closed.   The mouth of the electrode vessel
to which the membrane is applied is 6.5 mm. in internal diameter.

Serum was obtained by bleeding the animals, defibrinating, and centrifugating
the blood; it contained sufficient hemoglobin to give a faint or deeper rose tint.
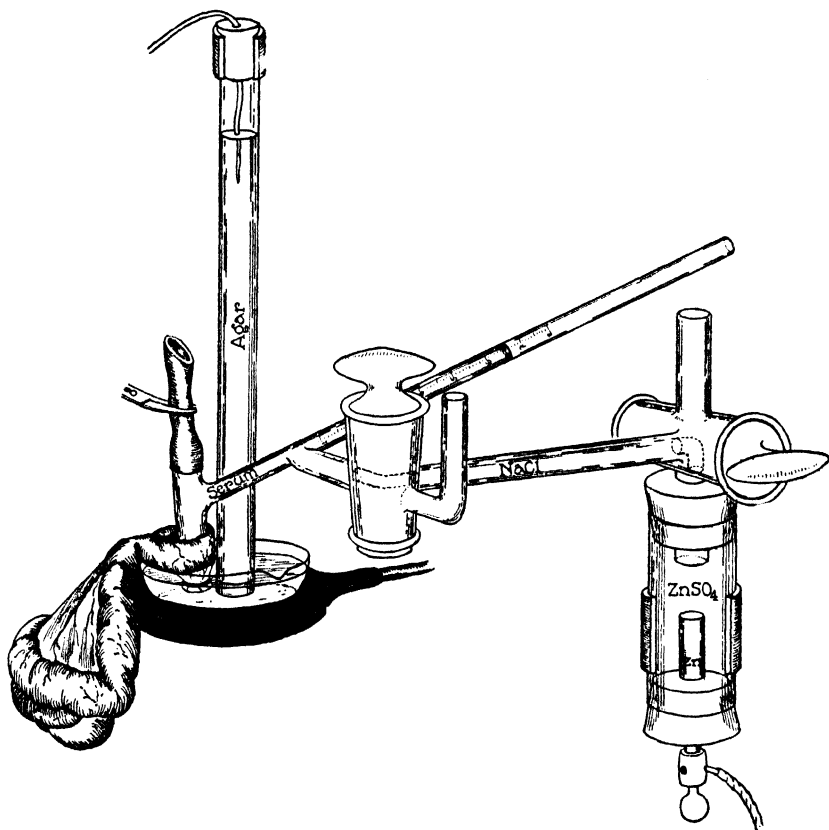
FIG. 5. Arrangement for study of electroendosmotic transport using whole
blood serum and mesentery of living animal.   See text.

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[5] Replacement of this L-way cock by a T-way cock adapts the apparatus also
to the determination of the H ion reversal points of membranous tissues.   This
form of the apparatus may be had from Arthur H. Thomas Company of Phila-
delphia.

The serum from animals fasted before use was clear.   If the animals had been
newly fed the serum was turbid even after prolonged centrifugation, presumably
due to lipemia.   Dark-field examination of such turbid serum showed myriad
bright spherical objects ranging from the limit of visibility to droplets of a micron
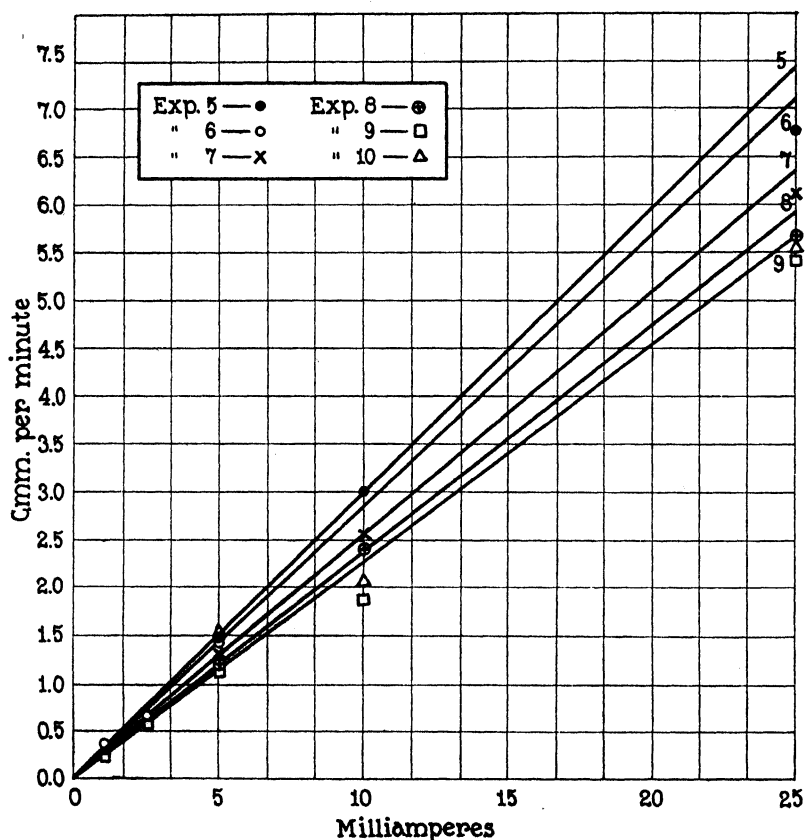
FIG. 6. Rates of transport of liquid plotted against current strengths.   Fluid
bathing membranes, undiluted dog blood serum.   Experiment 5, living dog mesen-
tery; Experiment 6, living dog mesentery; Experiment 7, dog mesentery, post
mortem; Experiment 8, dog pleura, post mortem; Experiment 9, dog pericardium,
post mortem; Experiment 10, dog pericardium, post mortem.

or more in diameter.   With the mesenteries of the living animals the fresh serum
of another animal of the same species was used as perfusing fluid; precautions were
taken against injury of the living mesentery.   With membranes used post mortem
the serum of the animal from which the membrane came was used.

In general the points obtained with current strengths up to 10 milliamperes are seen to lie satisfactorily along straight lines passing through the origin (Fig. 6).

TABLE II.

Experiment No.	Serum.		Membrane.	State of animal.	Current strengths (in order used).	Rate of transport in c. mm. per min. per milliampere.
	From	State.				
					milli-amperes	
5	Dog B.	Clear.	Mesentery, Dog C.	Living.	10, 5, 25	0.30
6	" D.	"	" " E.	"	5, 2.5, 1	0.28
7	" C.	"	" " C.	Dead 23 hrs.	5, 10, 25	0.25
11	" A.	Turbid.	" " A.	" 3 "	25	0.27
9	" F.	Clear.	Pericardium, Dog F.	" 3 "	5, 2.5, 1, 10, 25	0.23
11	" A.	Turbid.	" " A.	" 1½ "	25	0.23
8	" E.	Clear.	Pleura, Dog E.	" 18 "	5, 10, 25	0.24
12	" A.	Turbid.	" " A.	" 22 "	25	0.19*
13	Cat A.	Clear.	Mesentery, Cat. B.	Living.	25	0.22
14	" C.	Turbid.	" " D.	"	25	0.24
15	" C.	"	" " C.	Dead 7 hrs.	25	0.22
16	" B.	Clear.	Pericardium, Cat B.	" 4 "	25	0.26
17	" C.	Turbid.	" " C.	" 6 "	25	0.23
18	" B.	Clear.	Pleura, Cat B.	" 2 "	25	0.28
19	" D.	Slightly turbid.	" " D.	" 2 "	25	0.24
Average...						0.25

* The site of Experiment 12 seems to have been pleura from the anterior mediastinum rather than from the fibrous sheet between apex of pericardium and diaphragm ordinarily used. The section showed strands of atrophic thymus tissue and cysts filled with a coagulum between the pleural leaves.

Exceptions are the points plotted in triangles and the 10 milliampere point in Experiment 9. The points in triangles are, for reason not understood, so irregular that no attempt has been made to draw a line through them; they are not included in Table II.

All of the 25 milliampere values and the 10 milliampere point of Experiment 9 are lower than expectation. The disturbing factor is not known. The temperature of the serum bathing the membrane was raised a few degrees by the passage of the 10 and 25 milliampere currents, and this may have been of influence. Liquid flow may have become turbulent with the higher current strengths. However, the mean departure of observed values at 25 milliamperes from the straight lines amounted to only 5.4 per cent. The values of transport rate given in Table II for the experiments plotted in Fig. 6 are the slopes of the straight lines. For the experiments in which only 25 milliampere points are available the tabulated values for transport rate are probably about 5 per cent too low.

No certain correlation was detected between rate of transport and the thickness of the several membranes; this is in harmony with other electroendosmotic experiments.[6]

DISCUSSION.

A number of animal membranes have been shown to be negatively charged relative to their environing medium when that medium is blood serum or other buffer of neutral or slightly alkaline reaction. The existence of this electric potential difference necessitates that the liquid in the membrane pores should tend to move toward the cathode when the membrane is traversed by an electric current. The rate of liquid flow has been shown to be proportional to the current strength and to amount when serum is used to 0.2 to 0.3 c. mm. per minute per milliampere.

The functional activity of glands[7] and muscles is known to be accompanied by electric current flow, and numerous other sources of current in the body are either known or may be confidently inferred from analogy with non-living systems. The suggestion has already been made[8] that the action current of glands might influence the

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[6] von Smoluchowski, M., in Graetz, L., Handbuch der Elektrizität und des Magnetismus, Leipsic, 1914, ii, pt. 2, 380.

[7] Hermann, L., and Luchsinger, B., *Arch. ges. Physiol.*, 1878, xvii, 310. Bayliss, W. M., and Bradford, J. R., *J. Physiol.*, 1885, vi, p. xiii; 1886, vii, 217. Bradford, J. R., *J. Physiol.*, 1887, viii, 86. Cannon, W. B., and Cattell, McK., *Am. J. Physiol.*, 1916, xli, 39. Gesell, R., *Am. J. Physiol.*, 1918–19, xlvii, 411.

[8] Mudd, S., and Mudd, E. B. H., *J. Bact.*, 1924, ix, 163.

process of secretion. Consideration of the facts of the preceding paragraph, the writer believes, endows this possibility with a degree of probability amounting almost to certainty. For in such a system, in which liquid is being transported through capillary channels which are at the same time the site of an "action current" the electric current must at least modify if it does not control the liquid flow. Knowledge as to whether the electroendosmotic effect plays a major or minor part will have to await further study of the orientation and magnitude of the electric disturbances.

A number of the experiments here reported were performed by my technical assistant, Mr. Leo S. Hrdina.

SUMMARY.

The rate of electroendosmotic flow through dog and cat pericardia is found to be proportional to the current strength. The plots of current strengths against volumes of liquid transported in unit time are, in the better experiments, straight lines passing *through the origin;* the slopes of the lines are characteristic of the several systems.

Data on transport rate with buffers of different specific resistances showed the following phenomena:

1. Decrease of the observed transport rate to a minimum between σ values of 95 and 60 ohms.

2. Changes in the membrane markedly affecting transport rate, at conductivities and osmotic pressures close to those of the blood.

3. Polarization of the membrane during the passage of current.

The mean rate found for electroendosmotic transport across dog and cat serous membranes bathed in serum has been 0.19 to 0.30 (average, 0.25) c.mm. per minute per milliampere.[1] The best experiments with dog serum and the living mesenteries of dogs under ether gave a mean rate of 0.29 c.mm. per minute per milliampere.

These data, together with data from other sources, are believed to indicate a probability approaching certainty that electroendosmotic effects are a factor in glandular secretion.

GALVANIC STIMULATION OF LUMINESCENCE IN PELAGIA NOCTILUCA.

BY A. R. MOORE.[*]

(From the Physiological Laboratory of Rutgers University, New Brunswick, and the Zoological Station, Naples, Italy.)

The physiological evidences of excitation are muscle contraction, gland secretion, and luminescence. As a result of electrical stimulation Panceri[1] obtained luminescence in various light-producing cœlenterates. Recently E. B. Harvey[2] has studied galvanic stimulation of luminescence in *Noctiluca*, but was unable to discover any polar effects. I found, however, that the ctenophores *Mnemiopsis* and *Beroe* gave clear results with polar stimulation when a small current of a few milliamperes was passed through them.[3] On the make, a luminescent glow lasting several seconds occurs at the anode; and in *Mnemiopsis* a break flash can frequently be observed at the cathode. It should also be noted that in these two forms muscle contraction goes hand in hand with the luminescent response; *i.e.*, contraction of the musculature on the anodal side occurs at the make of the current. These facts serve to render it clear that the two ctenophores studied react to the electric current according to a reversal of the law of Pflüger. Now Pflüger's law has been assumed to be universal in its application, and an explanation has been sought in the field of ion effects.[4] Specifically, stimulation at the cathode on the make has been referred to the heightened irritability conferred by the excess of Na ions which collects at the cathode as a result

[*] Occupant of the Table of the American Association for the Advancement of Science, Naples, 1925.

[1] Panceri, P., *Ann. sc. nat. zool.*, 1872, xvi, series 5, September.

[2] Harvey, E. B., *Carnegie Institution of Washington, Pub. 251*, 1917, 245.

[3] Moore, A. R., *Proc. Soc. Exp. Biol. and Med.*, 1924-25, xxii, 80.

[4] Loeb, J., The dynamics of living matter, New York, 1906, 102.

of the flow of the current. This explanation can serve for the reversed Pflüger's law in either of two ways. First, the ionic conditions of stimulation may be reversed, in which case stimulation would be due to Ca and not to Na ions. In the second place, the locus of the action of the ions in stimulation may be on the side of the membrane opposite to that in the frog nerve. As to the first point, I have shown that *Mnemiopsis* is stimulated to luminescence by Ca, Sr, and Ba ions but not by Na and Mg ions.[5] I also found that the ion effect took place at the water-protoplasm boundary and not within the cells of the organism, because stimulation by the electric current occurred at the anodal face even when that was a cut surface.

With a view to obtaining further information on the galvanic stimulation of luminescence I worked with specimens of the medusa *Pelagia noctiluca*.[6] At the outset I found them less sensitive to the electric current than the ctenophores. For this reason I used platinum electrodes to carry the current into the trough. Non-polarizable electrodes were, however, tried and found to give concordant results, but the luminescence excited was faint because of the weakness of the current.

If a specimen of *Pelagia* is put into a rectangular glass dish containing sea water and a current of 200 ma. passed through, a glow occurs along the margin on the anodal side of the animal. In very sensitive specimens the luminescence spreads from this region like a blush over the whole bell. The glow continues during the flow of the current and ceases at the break. Under certain conditions there is a secondary glow on the cathodal side on the make and during the flow of the current.

It was first attempted to answer the question: Does the electric current produce its effect directly by acting on the luminescent

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[5] Moore, A. R., *Am. J. Physiol.*, 1925, lxxii, 230.

[6] 2 years ago Heymans and I[7] recorded the inhibiting effect of light on the luminescence of *Pelagia* and a day-night rhythm in luminescence. This year I failed to find either of these effects. Even exposure to strong sunlight for half an hour did not appreciably reduce the luminescence which appeared upon stimulating the animal in the dark. I have no idea how to account for such an extraordinary difference in behavior during the two seasons.

material contained in the cells, or indirectly by producing excitation in non-luminous tissue such as nerves and ganglion cells which in turn convey the impulse to the luminescent organs?

As a first step in the analysis, it was necessary to determine what effect, if any, the electric current exerted upon the luminescent material apart from the animal. Some of the luminescent slime was collected in sea water, put into a watch-glass, and the current passed through. On the make and during the flow of the current there was a bright glow at the cathode. Now there are two effects of the current either of which causes the photogenic granules to glow. One of these is the movement of the gas bubbles at the pole, which is sufficient mechanical stimulation to cause a very faint light. In the second place, the alkali which collects at the cathode is an important factor since hydroxyl ions are effective in causing the luminescent material of *Pelagia* to glow.[7] The phenomenon is therefore fundamentally different from the one described by Harvey.[8] He found that if the current were passed through a solution containing oxyluciferin and luciferase, the oxyluciferin is reduced in contact with the cathode and reoxidized in the vicinity of the cathode but that hydroxyl ions inhibit this reaction.

Since, in *Pelagia*, the luminescent material in solution glows only at the cathode, while the animal glows at the anode during the passage of the electric current, it is necessary to conclude that in the latter case the stimulation to luminescence by the current is indirect, in that the current acts on non-luminescent structures which transfer the impulse to the luminescent cells. It was also found in the cases where cathodal stimulation of the animal occurred that the animal lay very close to the cathode, so close as to be acted upon by the alkali formed by the current. That alkali will cause luminescence of the animal was shown by letting fall a drop of N/10 NaOH in sea water near the rim of a specimen of *Pelagia* swimming in sea water. The result was a luminescent glow in the region involved. N/10 acetic acid applied similarly did not have any effect. These facts suggest that the cathodal glow is caused by hydroxyl ions formed at the cathode.

[7] Heymans, C., and Moore, A. R., *J. Gen. Physiol.*, 1923–24, vi, 273.

[8] Harvey, E. N., *J. Gen. Physiol.*, 1922–23, v, 275.

The question has often been raised as to whether luminescence is not a by-product of muscular contraction. The two phenomena occur together in $CaCl_2$ poisoning. In pure $CaCl_2$ solution *Pelagia* is hypersensitive, the musculature becomes systolic and spontaneous luminescence spreads over the whole bell.[7] But in a solution of KCl these effects are separate, for the reason that while potassium causes relaxation of the musculature, luminescence is excited throughout the bell and tentacles. Furthermore, the galvanic current causes swimming pulsations of the bell (rhythmical contraction and relaxation of the musculature) but a continuous glow. For these reasons it must be concluded that luminescence is not dependent upon the contraction of muscles but is an independent phenomenon which occurs as the result of primary stimulation.

Last year I found, in *Mnemiopsis*,[5] that a transverse incision in the animal resulted in the formation of an additional anode at the cut surface. In *Pelagia*, however, no such result was obtainable. Even when the animal was cut in two completely, there was no glow from the cut surface of the bell when this faced the anode. In fact it could be shown that galvanic stimulation occurred only along the margin of the umbrella, for if the margin were cut off, the bell gave no response to the current. The isolated margin, however, gave the usual galvanic reaction, namely, luminescence at the anode. This result serves to indicate that the current acts upon nervous elements in the margin of the bell.[9] In this respect the experiment recalls the earlier one of Loeb and of Loeb and Budgett on *Amblystoma*,[10] in which they found that the skin secretion took place at the anode during the passage of the galvanic current. They also proved the dependence of the phenomenon on the nervous system, since section of the cord alone caused the formation of an additional anode. They found that NaOH when applied to the skin caused secretion, and concluded that the current acted by means of the positive ions in the medium surrounding the animal. These ions migrate toward the cathode. They would therefore impinge upon the anodal side

[9] Loeb, J., *Am. J. Physiol.*, 1899–1900, iii, 383. Romanes, G. J., Jellyfish, starfish and sea urchins, New York, 1885, 65.

[10] Loeb, J., *Arch. ges. Physiol.*, 1897, lxv, 308. Loeb, J., and Budgett, S. P., *Arch. ges. Physiol.*, 1897, lxv, 518.

of the animal and stimulate secretion there. This is identical with the mechanism which I have suggested to explain anodal stimulation of luminescence in *Mnemiopsis* and *Beroe*.[5] But stimulation at the cathode in *Pelagia* is due to hydroxyl ions. There are, therefore, two kinds of ionic stimulation, namely (1) anodal, which is referable to the blocking of positive ions by the tissue on that side, and (2) cathodal stimulation, when the animal is near the cathode, is due to the diffusion of alkali (hydroxyl ions) outward from a region of high concentration.

<center>CONCLUSIONS.</center>

1. *Pelagia noctiluca* responds to galvanic stimulation by a luminescent glow at the anode. If placed near the cathode a secondary glow occurs also on the cathodal side.

2. The luminescent slime of *Pelagia* when subjected to the galvanic current glows around the cathode. This is referred partly to the movement of hydrogen bubbles, but in the main to the alkali formed at the cathode.

3. The cause of galvanic stimulation in *Pelagia* is ionic. (1) Anodal stimulation is referred to the blocking of positive ions by the tissue on that side. (2) Cathodal stimulation, when the animal lies near the cathode, is due to the diffusion of alkali outward from a region of high concentration (the cathode).

4. Only the margin of the bell is excited to luminescence by the galvanic current. It is therefore concluded that nervous elements are the seat of excitation.

5. Luminescence is not a result of muscular contraction, since K ion causes relaxation of musculature but a continuous luminescent glow in *Pelagia*. The galvanic current causes pulsations of the bell (contraction and relaxation of the musculature) but a continuous glow.

EVIDENCE OF A FACTOR ASSOCIATED WITH ACTIVELY FUNCTIONING TISSUES WHICH GIVES TO SUGAR-CANE PLANTS RESISTANCE TO THE INVASION OF FUNGI AND OTHER MICROORGANISMS.

By H. ATHERTON LEE.

(From the Laboratories of Pathology and Physiology, Experiment Station of the Hawaiian Sugar Planters' Association, Honolulu, T. H.)

It is with hesitation that one discusses in publication such a broad subject as the resistance of plant tissues to the invasion of microorganisms, based on what seem to be very simple observations. It is reassuring, however, to recall the fundamental and broad conclusions of Jacques Loeb, drawn from his very simple quantitative experiments with *Bryophyllum*. If one recalls also the fundamental conclusions in animal physiology obtained by Claude Bernard from pathological cases, confidence is regained to present the following evidence; an indication also that in some instances pathological cases in plants may contribute to an advancement in the knowledge of plant physiology.

The Normal Development of Sugar-Cane Roots.

Sugar-cane is propagated on a plantation scale by cuttings of the cane stem. These cuttings, usually consisting of three or four nodes, are planted horizontally in the ground, and rootlets arise from areas at each node known as root bands; subsequently, an aerial shoot develops from the single bud which exists at each node. Normally such an aerial shoot feeds upon the parent stalk cutting for a period of 1 or 2 months; during this period the cutting has put forth small roots from the root bands of the nodes so that the aerial shoot receives its mineral food through the cutting. The aerial shoot does not form its own roots for a considerable period after germination.

In a month or two, depending on environmental factors, the aerial shoot normally forms its own roots and the seed cutting gradually

is invaded by fungi and other microorganisms and gradually loses its function in supporting the aerial shoot.

This development is shown in Fig. 1; the letter N denotes the nodes, B the buds, one at each node; above each node is a flat narrow band
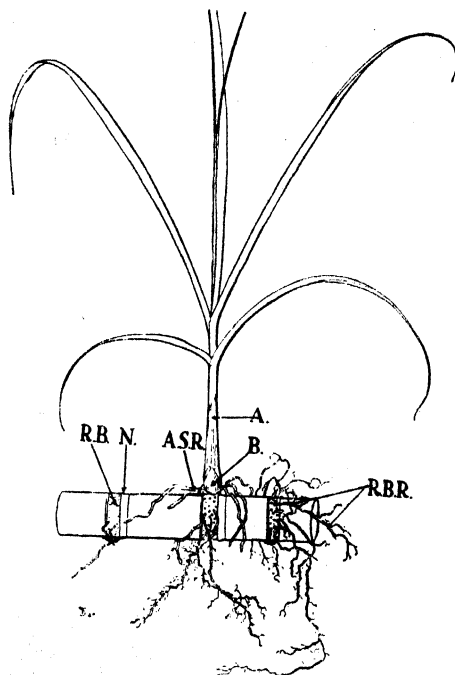
FIG. 1. A diagrammatic illustration of the parts of a sugar-cane plant arising from a stem cutting. N denotes a node of which there are three in the illustration. At each node is a root band indicated by the letters $R.B.$ From such root bands the cutting sends out roots which are labelled $R.B.R.$ in the illustration. At each node is a single bud denoted $B.$, and in the illustration the central one of these has germinated resulting in the aerial shoot $A.$ The aerial shoot, after some time for its development, forms its own roots indicated by the letters $A.S.R.$, which are independent of the roots formed from the cutting. Drawing by Twigg Smith.

known as the root band shown in the figure as $R.B.$ From the root bands, $R.B.R.$ shows the root band roots developing. A shows the aerial shoot and $A.S.R.$ the aerial shoot roots which are formed at a considerable period after germination has taken place.

A Suggestive Pathological Condition.

In Hawaii a disease of sugar-cane known as Pahala blight occurs. In some of the phases of this disease the aerial shoot seems incapable of forming its own roots and feeds through the seed cutting; instances have been observed in which the seed cutting functioned for 12 months after planting, with no formation of roots by the aerial shoots. The seed cutting in such cases did not succumb to soil fungi such as invade seed cuttings under usual conditions. Non-blighted, healthy stools of cane adjacent to affected stools, however, developed roots from their aerial shoots and in such cases the seed cuttings were invaded with microorganisms, the tissues were entirely rotted, and the cuttings no longer functioned in supporting growth of the aerial shoot. Such observations were uniform on some twenty or thirty shoots of cane affected with Pahala blight which were examined, and an almost equal number of healthy stools dug up and inspected.

The question arises in these cases, of course, whether the freedom from invasion by microorganisms was a cause or an effect; this question is eliminated by a coincident experiment with normal cane mentioned in the following paragraphs.

An Experiment with Normal Cane.

At this Experiment Station a study was being conducted at the same time on the function of the roots from the root bands of seed cuttings in connection with the growth of aerial shoots. In potted cane plants the aerial shoots were maintained free from the soil and all roots from the aerial shoots were pruned off as they arose. The aerial shoots were thus forced to draw their mineral supply through the seed cuttings and the roots from the root bands of the seed cutting. At the end of 6 months it was found that the aerial growth took place just as well in those plants forced to feed through the seed cutting as in normal plants as controls under identical conditions, which formed their later roots as usual from the aerial shoots. The roots of the plants were then washed out and examined.

In all cases where no roots were allowed to develop from the aerial shoots, the plant had continued to be supported from the seed cutting and the invasions of fungi and other microorganisms in these func-

tioning cuttings were inhibited. In the control cane plants, where roots had been allowed to develop normally from the aerial shoots, the seed cuttings were entirely invaded by microorganisms and had rotted away. This difference in the two classes of cuttings is shown
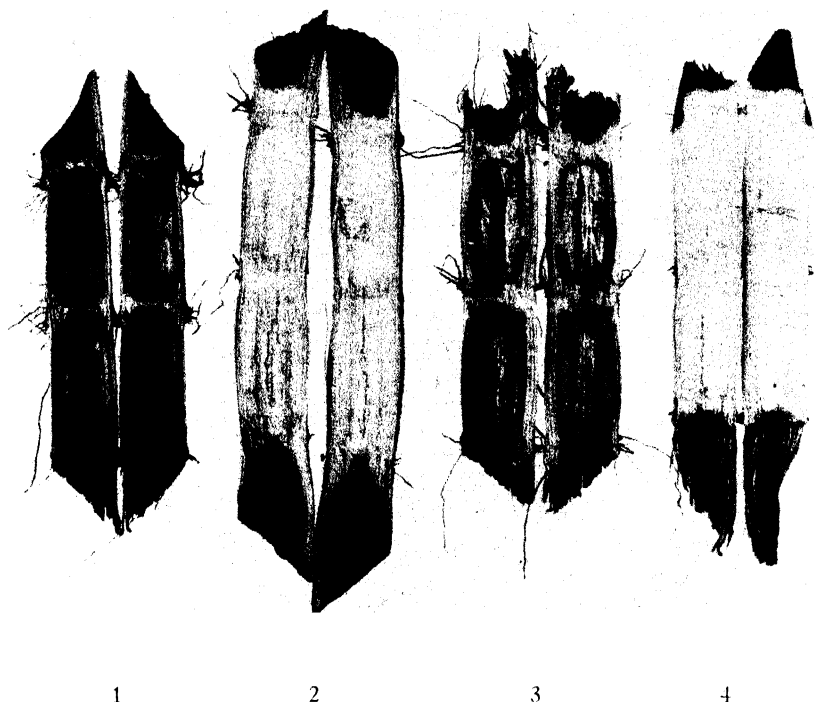
Fig. 2. Cuttings 1 and 3 are from plants in which aerial shoots were allowed to form their own roots; Cuttings 2 and 4 are from plants under identical conditions, the roots of which, however, were excised at their inception, thus forcing feeding through the cutting and the roots from the root cutting.   Cuttings 2 and 4, therefore, were actually functioning and as the photograph shows completely resisted invasions of fungi and microorganisms such as invaded Cuttings 1 and 3. The photograph was taken about 200 days after the planting of the cuttings.

in Fig. 2.   In the figure, seed Cuttings 1 and 3 are from stools in which the roots from aerial shoots were allowed to develop normally and function in the support of the plant. Cuttings 2 and 4 are from stools in which aerial shoot roots were pruned off as they developed,

thus causing the aerial shoots to depend for their support on the seed-piece cuttings and the roots developed from the root bands of the cuttings. There were six replications of plants with excised roots, and controls.

DISCUSSION.

The conclusion which would seem to be deduced from these phenomena is that there is a factor, or there are several factors, in actively functioning plant tissues, or at least in the tissues of sugar-cane plants, which give to such tissues resistance to the invasion of low-grade fungi and other microorganisms. A review of the discussions on resistance and immunity in plants by Appel (1), Biffen (2), Butler (3), Cook and Taubenhaus (4), Freeman (5), Orton (6), and Stakman (7), shows no previous clear-cut evidence in the literature pointing to such a conclusion, although undoubtedly there has been belief in such resistance by many students in the plant industries and students of plant pathology.

In explanation of this resistance one may advance very simple physical factors such as the greater turgor of actively functioning cells as compared to those in non-functioning tissues. Other explanations may lie in the realm of substances formed by the aerial shoots which normally flow to the roots giving them resistance to fungus invasion; when the normal roots from the aerial shoots are inhibited, such substance may, in passing to or through the seed cutting, contribute to its resistance. Such explanations, however, are at present entirely in the realm of conjecture; there remains, however, the seemingly clear-cut evidence that there is a factor in actively functioning plant tissues which causes resistance to the invasions of low-grade microorganisms under conditions in which non-functioning tissues are quickly invaded. Many of the conjectures as to the reason for this could be readily put to proof in institutions devoted to non-commercial research.

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THE TRANSPORT OF GAS BY THE BLOOD OF THE TURTLE.

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The present paper contains an account of the blood of the turtle, considered as a system for carrying oxygen and carbon dioxide. It will be found of interest in showing how greatly the blood of these animals may differ from that of man—and to what factors the dissimilarities are due. The meaning of the chemical characteristics of the turtle's blood in terms of the peculiarities of these animals is not clear at present and must await further experimentation on their respiratory habits.

Practically all of the observations were made on the blood of *Pseudemys concinna.* Blood was drawn from the left aortic arch or left pulmonary artery through a hole trephined in the carapace in the mid-line just anterior to the center. Coagulation was prevented by the use of powdered sodium oxalate. In a few cases blood was drawn under oil and the gases determined directly; but in most of the experiments it was brought into equilibrium with gas mixtures in tonometers. The blood was put on ice as soon as drawn, and so kept until placed in the tonometers. It was considerably less viscous than human blood, and the cells showed a tendency to settle very rapidly. For this reason it was necessary to use special care to stir the blood thoroughly before taking samples.

A temperature of 25°C. was selected for the equilibration of the blood with the gas mixtures, inasmuch as this represents a temperature within the physiological range of the turtles studied and can be maintained readily in a thermostat. Samples of about 5 cc. of blood were brought into equilibrium with 250 cc. of gas mixture by rotating for 20 minutes or more in a water bath. The blood gases were then determined by the modified Van Slyke method (1). The oxygen was not reabsorbed, 1.36 volumes per cent being subtracted from the final reading as representing the nitrogen in the blood. Duplicate determinations were made whenever possible. The percentages of the gases in the tonometers were then accurately determined by means of the Haldane apparatus, and their partial pressures estimated by the formula given by Bock, Field, and Adair (2).

Where true plasma was required, the equilibration was carried out as usual,

and one portion was used for the determination of the carbon dioxide content of the whole blood. The remainder was then centrifuged under oil for 5 minutes, and the plasma was immediately transferred to the Van Slyke apparatus and analyzed for carbon dioxide.

Character of the Blood.

The blood obtainable from single turtles, weighing from 0.87 to 2.6 kilos, varied from 30 to 70 cc. The corpuscular volume of each blood was determined by means of the hematocrit, and was found to vary between 9.4 and 22.3 per cent of the volume of whole blood. The observations were made during December and January. It is perhaps worthy of note that the lower percentages of cells were found chiefly in turtles which had been kept for a considerable length of time in our animal house, while the higher percentages were chiefly in those examined when freshly received from the dealer about January 1. The oxygen capacity of the blood was found to be correspondingly low and variable. The values are indicated in Table I, together with the hematocrit readings. The proportion between them is fairly constant. 1 cc. of cells will combine with nearly 0.5 cc. of oxygen. Bock, Field, and Adair (2) have shown that human blood having 40 volumes per cent cells has an oxygen capacity of 20 volumes per cent; i.e., that 1 cc. of cells will combine with 0.5 cc. of oxygen. The oxygen-carrying power of the red blood cells of the turtle per unit volume is almost exactly the same as that of human erythrocytes. In the alligator, on the other hand, Hopping (3) found that blood having a corpuscular volume of 14.5 per cent had an oxygen capacity of 12 volumes per cent. This indicates that 1 cc. of alligator's cells will combine with 0.83 cc. of oxygen and that their oxygen-carrying power is almost twice as great as that of human or turtle cells.

The carbon dioxide contents of two samples of blood as drawn from the efferent vessels of the heart were 92.4 and 80.3 volumes per cent. Because of the probable admixture of pulmonary and systemic venous blood in these vessels the exact significance of the figures is questionable.

The carbon dioxide capacity of the blood from various animals, when equilibrated with 42 ± 1 mm. carbon dioxide pressure at 25°C., was found to be 72.0, 77.1, 77.9, 83.8, 84.1, 85.9, 86.4, 88.6, 88.8,

TABLE I.

Cells.	O₂ capacity.	Ratio.
per cent	*vol. per cent*	
22.3	9.5	0.426
22.0	10.85	0.493
20.0	9.71	0.485
15.2	7.09	0.466
14.7	6.93	0.471
14.5	6.56	0.452
13.0	7.40	0.569
9.8	6.59	0.673
Average...		0.504
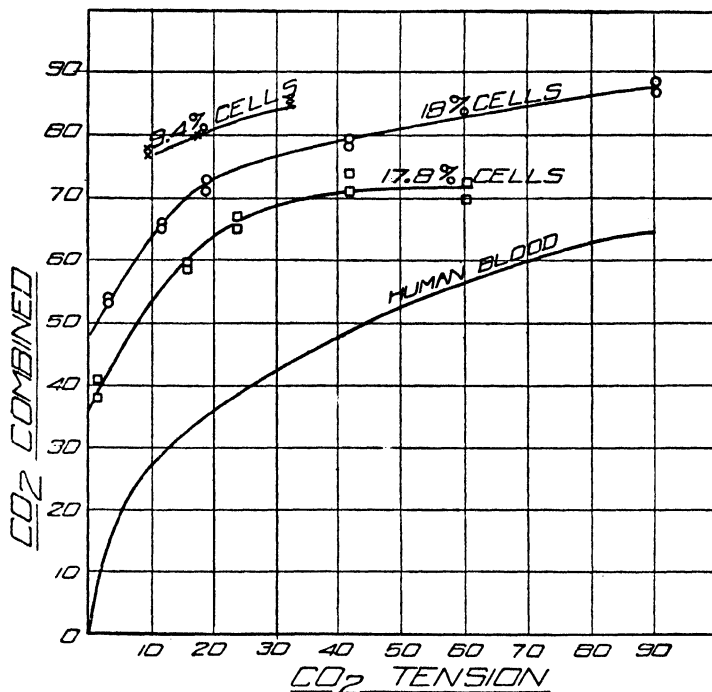
FIG. 1. Carbon dioxide dissociation curves of the oxygenated blood of *Pseud-emys concinna* equilibrated at 25°C. Partial pressures of carbon dioxide measured in mm. along the abscissa. Combined carbon dioxide measured in volumes per cent along the ordinate.

88.8, 91.2, 91.7, and 94.1 volumes per cent. These capacities are comparable to those found in turtles by Collip (4), amounting to 81.8 and 89.3 volumes per cent for whole blood and 80 volumes per cent for true plasma. Collip points out that these values are note-worthy as being high when compared with the other species of verte-brates which he studied.[1] The condition does not appear to be one generally characteristic of reptiles. Collip (7) finds a capacity of 45 volumes per cent in the garter snake (*Thamnophis sp.*). In the alligator Hopping (3) has observed carbon dioxide capacities of from 45.9 to 67.8 volumes per cent according to season. Nor is it due to a seasonal variation as we have found comparable values in turtles freshly taken in June.

The manner in which these extraordinary amounts of carbon diox-ide are carried in the blood of the turtle is revealed by the dissocia-tion curves of oxygenated blood shown in Fig. 1. Two features mark these curves as distinctive; (1) the greatest reduction of the carbon dioxide pressure attained fails to remove a residuum of 35 to 50 volumes per cent of combined carbon dioxide, and (2) the general slope of the curves is decidedly flatter than is usual in human blood. Since the completion of our experiments, Wastl and Seliškar (8) have published dissociation curves for the frog (*Rana catesbiana*) which share these characteristics with the turtle, but have a gen-eral level intermediate between that of this reptile and that of man. The interpretation of these characteristics, from the view-point of the blood as a physical system, is not difficult. Under or-

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[1] Jolyet (5) found 40 to 54 volumes per cent in the arterial blood of the turtle (*Emys europaea?*). In the arterial blood of the sea turtle, *Thalassochelys caretta*, Spallitta (6) observed 49.01 to 53.65 volumes per cent carbon dioxide. The dis-crepancy between these values and those observed by Collip and ourselves we believe may be attributed to the fact that these workers employed the blood gas pump for their analyses. In two cases we have carefully pumped off and expelled all the carbon dioxide possible with the Van Slyke apparatus before adding the lactic acid to free the combined carbon dioxide. In these cases we have found 45.5 and 59.1 volumes per cent of carbon dioxide which had not been removed by evacuation. If such values be subtracted from the carbon dioxide contents of these bloods when equilibrated with 44 mm. carbon dioxide pressure, one obtains 31.0 and 26.8 volumes per cent as the carbon dioxide removed by evacuation.

dinary conditions this blood contains a large quantity of carbon dioxide combined as bicarbonate and at the same time a relatively small percentage of corpuscles. In human blood, as the carbon dioxide pressure is reduced, the bicarbonate is decomposed and the base combines with the hemoglobin and phosphate of the corpuscles or with acids set free by an exchange of ions between the plasma and these corpuscular buffers; see Joffe and Poulton (9).[2] In the turtle blood the base-binding capacity of the available corpuscular buffers is deficient for this purpose and suffices to set free only about one-half of the carbon dioxide which is held in combination at physiological levels. Further, since the slope of the dissociation curve is a measure of the available base-binding, or yielding, capacity of the blood and as this is provided in large part by the hemoglobin and phosphate of the corpuscles (Van Slyke (10)) the pronounced flatness of the curves is also attributable to the low corpuscular content.

The validity of the foregoing explanation is demonstrated by the following observations. The quantity of carbon dioxide taken up by several samples of blood between two fixed hydrogen ion concentrations is given in Table II. It is clear that this measure of the buffer value of the bloods is roughly proportional to the number of red corpuscles which they contain. Furthermore, by increasing the corpuscular concentration artificially it may be shown that the slope of the carbon dioxide dissociation curve is increased and that the quantity of carbon dioxide which may be given off under a reduced partial pressure is considerably amplified. To do this blood containing 17.5 per cent corpuscles was centrifuged under oil at a tension of 33 mm. of carbon dioxide, about two-thirds of the plasma was pipetted off, and the remaining plasma was again mixed with corpuscles. The resulting blood had a corpuscular volume of 44 per cent, approximating that of human blood. The dissociation curve of this blood is shown in Fig. 2, in which is included for comparison a curve of human blood, one of turtle blood containing 18 per cent of red corpuscles, and one of separated turtle plasma. The latter curve was obtained from the plasma of blood equilibrated with a gas mixture containing 49.1 mm. carbon dioxide in

[2] Collip (4) has shown that such an ionic exchange occurs between the corpuscles and plasma of a number of animals, including the turtle.

TABLE II.

Corpuscles.	Buffer action.*	Ratio.
per cent	vol. per cent	
0.0†	4.5	
9.4	6.0	0.64
9.7	7.0	0.72
17.8	14.0	0.78
18.0	11.0	0.61
19.8	14.5	0.73
44.0‡	20.0	0.45

* Change in carbon dioxide content between pH 7.92 and 7.58.

† Plasma separated from corpuscles when in equilibrium with 49.1 mm. carbon dioxide.

‡ Concentrated artificially when in equilibrium with 33 mm. carbon dioxide.
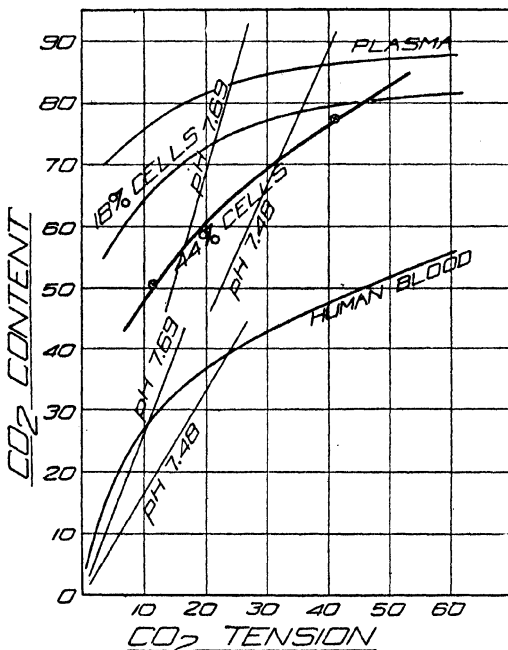
FIG. 2. The three upper curves are carbon dioxide dissociation curves of the blood of *Pseudemys concinna* equilibrated at 25°C. The lower curve is of human blood (A. V. B. (2)) equilibrated at 37.5°C. Partial pressures of carbon dioxide measured in mm. along the abscissa. Combined carbon dioxide measured in volumes per cent along the ordinate.

air, and centrifuged under oil, without loss of carbon dioxide. Here the effect of the corpuscles is entirely absent, and it is seen that the amount of carbon dioxide given off when the partial pressure is reduced is correspondingly diminished. A more exact comparison may be made by comparing the carbon dioxide combined by the bloods when a fixed change in pH is produced by an increase in the carbon dioxide pressure—a manipulation which will cause a corresponding shift in the ratios of the weak acids and their salts which are buffering both systems. In this comparison allowance must be made for the different temperatures at which the bloods are studied. The method of estimating the pH of the turtle blood is described below. For a change in pH from 7.69 to 7.48 the values in Table III are obtained.

TABLE III.

	Concentrated turtle blood (44 per cent cells).	Human blood (40 per cent cells).
pH	*vol. per cent*	*vol. per cent*
7.48	72	38
7.69	58	27
Difference.............	14	11

The Hydrogen Ion Concentration of the Blood Plasma.

The pH of the blood plasma may be estimated from the data of the carbon dioxide dissociation curve by means of Hasselbalch's (11) equation;

$$pH = pK_1 + \log \frac{BHCO_3}{H_2CO_3}$$

For this purpose it is necessary to fix upon values for pK_1 and α, the solubility coefficient of carbon dioxide, which are appropriate for this blood and the conditions under which it was equilibrated.

The value of pK_1 for horse blood adopted by Van Slyke, Wu, and McLean (12) is 6.12 at 38°C. This value may be corrected for temperature by the addition of 0.005 for each degree fall in temperature (Hasselbalch (11), Warburg (13)). A further small correction for the high concentration of bicarbonate in the turtle blood appears to be indicated by the experiments of Hasselbalch upon

sodium bicarbonate solutions. This is made by subtracting 0.03 from the value of pK_1. The value finally taken for turtle blood at 25°C. is $pK_1 = 6.15$.

The value of the solubility coefficient is much more profoundly altered by the lower temperature at which the turtle blood has been studied. Bohr (14) has given the values of α for water over a considerable range of temperatures. By interpolation α is 0.76 at 25°C. Bohr also gives values for α for plasma and whole blood at 15° and 38°C. At both these temperatures the solubility coefficient for plasma is 97.5 per cent of that for water, and the value for whole blood is 92 per cent of that for water. For turtle blood, which usually contains somewhat less than half as many corpuscles as the whole blood of mammals, we have selected a value 95 per cent of that of water, which at 25°C. gives $\alpha = 1.72$. Using these constants, calculations have given values of 7.54 to 7.42 for the pH of blood at 42 ± 1 mm. carbon dioxide tension. A sample of blood drawn directly from the aortic arch and analyzed by the dialysis method of Dale and Evans (15) has given a pH value of 7.5 $\pm$ 0.1.

The Effect of Oxygenation and Reduction.

The effect of oxygenation and reduction upon the carbon dioxide dissociation curve of the turtle blood is of interest from several viewpoints. In the first place it is not a foregone conclusion that the hemoglobin of all animals will exhibit the property of becoming a stronger acid upon oxygenation—thus augmenting its function as a buffer substance in a rather adaptive manner. Secondly, we have shown that the characteristics of the carbon dioxide dissociation curve are interpretable in terms of the concentrations of bicarbonates and corpuscles peculiar to the turtle blood. One may seek in the effects of oxygenation and reduction a further test of the adequacy of this conception of the specific characters of this blood. Finally, there is the question whether the magnitude of this phenomenon is such as to be of importance in the physiology of the living animal. A preliminary determination of the carbon dioxide dissociation curves of oxygenated and reduced blood showed that the difference between the two was extremely slight being about 2 volumes per cent at physiological tensions in blood containing 9.4 per cent corpuscles.

It therefore appears that while oxygenation and reduction affect the turtle dissociation curve and the human curve in the same way, the effect is too slight in the turtle to be of any practical importance, either in the transport of carbon dioxide or in the determination of the hydrogen ion concentration of the blood. In order to examine more closely the magnitude of this effect and its relation to the conditions in human blood, the turtle blood, concentrated to contain 44 per cent corpuscles, was reduced and equilibrated with mixtures
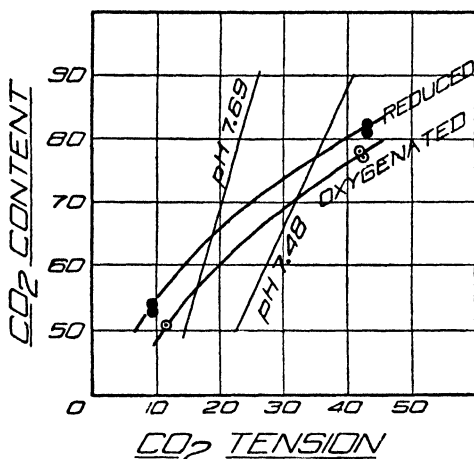
FIG. 3. Carbon dioxide dissociation curves of the blood of *Pseudemys concinna* concentrated to contain 44 per cent corpuscles, showing effect of oxygenation and reduction on carbon dioxide-combining power. Partial pressure of carbon dioxide measured in mm. along the abscissa; combined carbon dioxide measured in volumes per cent along the ordinate. Temperature 25°C.

of carbon dioxide and nitrogen. The results are compared with the findings for the oxygenated blood in Fig. 3. It is clear that with the concentration of the corpuscles there is no question that reduction increases the carbon dioxide-combining power of the blood. Reduced blood will now combine with about 7 volumes per cent more carbon dioxide without change in pH. This is approximately the difference between the oxygenated and reduced human blood over a similar range of hydrogen ion concentrations; in the case of human blood the difference is 6.3 volumes per cent (2). The recent work of Wastl and

Seliškar has demonstrated a distinct difference between the carbon dioxide dissociation curves of the oxygenated and reduced blood of the frog.

Distribution of Carbon Dioxide between Corpuscles and Plasma.

Determinations of the distribution of carbon dioxide between corpuscles and plasma were made by analyzing whole blood and true plasma at several tensions of carbon dioxide. The results are plotted
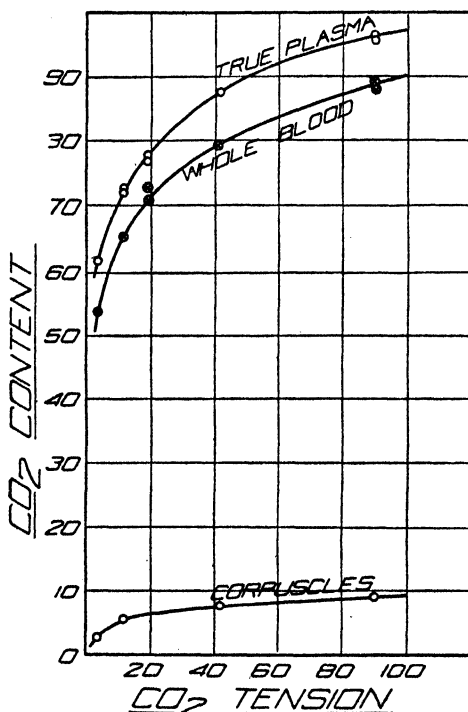
FIG. 4. Curves showing the distribution of carbon dioxide between the corpuscles and plasma in the blood of *Pseudemys concinna* at 25°C. Partial pressure of carbon dioxide measured in mm. of mercury along the abscissa. The upper curve is the volume of carbon dioxide combined with 100 volumes of true plasma at various carbon dioxide tensions. The middle curve represents the volume of carbon dioxide combined with 100 volumes of whole blood. The lowest curve shows the volume of carbon dioxide contained in the corpuscles of 100 volumes of whole blood.

in Fig. 4.   The carbon dioxide content of the plasma in 100 volumes of whole blood can be calculated, as described by Joffe and Poulton (9), by multiplying the content of true plasma by the fraction of plasma in whole blood.   If this quantity is then subtracted from the content of the whole blood, the difference will be the amount carried by the corpuscles.   In this case the fraction of corpuscles was 0.18 and that of plasma 0.82.   The result of this calculation is indicated by the lowest curve in Fig. 4.   From this curve it may be seen that a very small fraction of the carbon dioxide is carried by the cells. as indeed is to be expected from the small percentage of cells in the blood.   The carriage of carbon dioxide by the turtle corpuscles compares very favorably with that of human blood, however, when allowance is made for the difference in number of corpuscles.   From these figures we estimate that 100 cc. of turtle corpuscles at a carbon dioxide tension of 40 mm. are combined with 44 volumes of carbon dioxide.   At an equal tension 100 cc. of human blood corpuscles combine with only 29.2 volumes according to the calculations of Henderson, Bock, Field, and Stoddard (16).   This discrepancy in favor of the turtle blood may be attributed to two factors.   The first of these is the greater solubility of carbon dioxide at low temperatures.   In order to estimate the effect of this difference, it is necessary to compare the bloods at tensions such that the cells will contain the same concentration of carbon dioxide in solution.   The solubility coefficient of carbon dioxide in water is 0.555 at 38°C. and 0.76 at 25°C.   It may be supposed, therefore, that corpuscles at 38°C. will dissolve only $\dfrac{0.555}{0.76}$ or 73 per cent as much carbon dioxide as corpuscles at 25°C.   Since the amount of carbon dioxide dissolved is proportional to α times its partial pressure, human cells will dissolve as much carbon dioxide at 40 mm. carbon dioxide tension as turtle cells at $40 \times \dfrac{0.555}{0.76}$ or 29.2 mm. tension.   At these respective tensions 100 cc. of human corpuscles at 38°C. combine with 29.2 volumes of carbon dioxide, while 100 cc. of turtle corpuscles at 25°C. combine with 38 volumes.   There is thus still a difference of 9 volumes per cent in favor of the turtle cells.

The second point to be considered is that the concentration of

bicarbonate ion inside of the corpuscle may be expected to bear a rather definite ratio (determined by the Donnan equilibrium) to the concentration in the plasma. We have seen that the concentration of bicarbonate in the whole blood is exceptionally high when compared to the conditions in other animals. These considerations undoubtedly account for the high carbon dioxide-combining power of the turtle corpuscle. The exact ratio of bicarbonate ion within and without the corpuscle is of considerable interest inasmuch as it gives, theoretically, the proportion of all other diffusible ions on either side of the corpuscular wall, and in addition depends for its value on the excess of non-diffusible electrolyte within the corpuscle. The ratio has been designated by the symbol, r, by Van Slyke, Wu, and McLean (12), who define the concentrations in question in terms of the ratio of solute to the *volume of water* as solvent. We have calculated values for r on the assumption that 1 cc. of plasma contains 0.9 gm. of water, and that 1 cc. of corpuscles contains 0.63 gm. of water. These figures are round numbers taken from Van Slyke, Wu, and McLean's data on the horse blood. They are obviously not quite correct when applied to the turtle but may be justified for an approximate calculation on the ground that the figure for the plasma probably lies between 0.9 and 1.0 since the turtle blood is of lower osmotic pressure than that of the mammals. The figure for the corpuscles is open to greater doubt and in its justification we can only point out that the oxygen capacity of the turtle corpuscles indicates that the concentration of hemoglobin does not differ greatly from that of human erythrocytes. Using these values we estimate from the data in Fig. 4 that $r = 0.71$ at 41 mm. carbon dioxide. In oxygenated human blood at this tension, $r = 0.72$, according to the estimations of Henderson, Bock, Field, and Stoddard (16), while in the horse blood at 41 mm. carbon dioxide tension Van Slyke, Wu, and McLean find a value of 0.722. The agreement could not be better and shows that the high carbon dioxide-combining power of the turtle corpuscles may be accounted for strictly by the high bicarbonate content of the plasma if one assumes that the concentration of water and of non-diffusible electrolytes is the same in these cells as it is in the mammalian erythrocyte.

The Oxygen Dissociation Curve.

The form of the oxygen dissociation curve is illustrated in Fig. 5 by two typical examples equilibrated in the presence of 40 mm. carbon dioxide. For comparison is included with them the curve
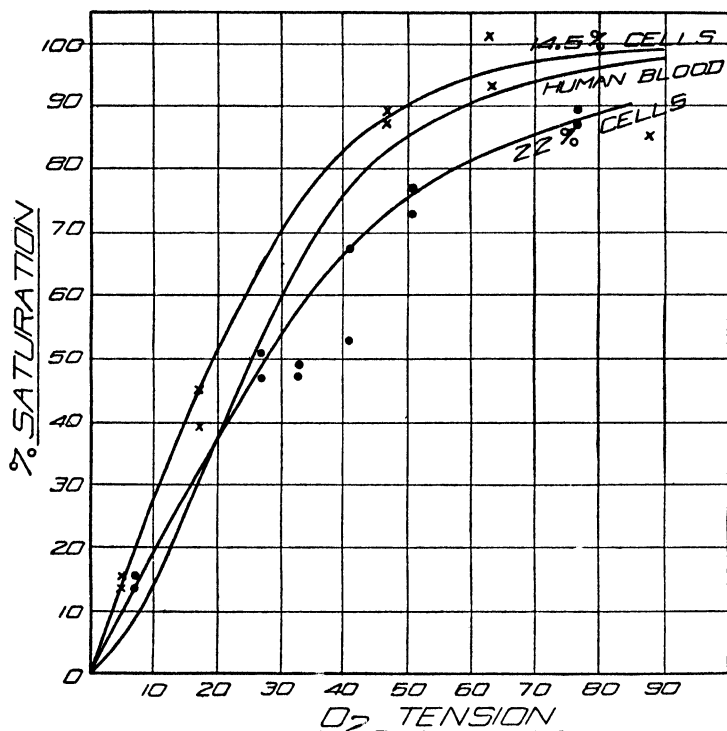
FIG. 5. Oxygen dissociation curves of the blood of *Pseudemys concinna*, equilibrated at 25°C. and in the pressure of 40 ± 1 mm. carbon dioxide. The corpuscular content is indicated and the curve for human blood (A. V. B. (2)) at 37.5°C. is included for comparison. Ordinates represent the per cent saturation of hemoglobin with oxygen. Partial pressure of oxygen measured in mm. of mercury along the abscissa.

of human blood at 40 mm. carbon dioxide pressure. In a general way and for all functional purposes these curves resemble that for human blood more than they resemble each other. There is, however, a distinct difference in the shape of the curves which

appears to characterize the turtle blood; *i.e.*, the S shape at low tensions is absent so far as one can judge from the measurements. The difference appears to be similar to that distinguishing the oxygen dissociation curve of the dog from that of man—which Barcroft and Camis (17) have attributed to specific differences in the electrolyte content of the blood.

The two curves illustrate the limits between which most of the other observations which we have obtained have fallen. In their exact position the curves appear to group themselves according to the percentage of corpuscles in the blood and to be independent of the carbon dioxide capacity as the figures in Table IV indicate.

TABLE IV.

Corpuscles.	O_2 pressure of $\frac{1}{2}$ saturation.	CO_2 capacity at 40 mm. CO_2 pressure.
per cent	*mm.*	*vol. per cent*
9.8	22	87.6
13.0	. 20	83.8
14.5	20	91.2
14.7	18	88.6
15.2	19	88.8
20.0	28	94.1
22.0	28	91.7
22.3	26	85.9

This observation would appear to bear some relation to that of Douglas, Haldane, and Haldane (18) who found that when the oxygen capacity of human blood is reduced because a part of the hemoglobin is saturated with carbon monoxide, the affinity of the remainder for oxygen is increased. These authors also comment upon the fact that with the reduction in the quantity of hemoglobin available for oxygen carriage the S shape of the dissociation curve tends to disappear. Two factors in the conditions under which these oxygen dissociation curves were made, differ from those usual in the standard curves for human blood. These are the lower temperature at which the equilibration was carried out in the case of the turtle and the comparatively high bicarbonate content of this blood. Other things being equal the lower temperature at which the turtle blood was studied should

favor considerably the combination of oxygen with hemoglobin and cause the dissociation curve to shift to the left. Furthermore at 40 mm. carbon dioxide pressure and 25°C. the pH of oxygenated turtle blood containing 90 volumes per cent carbon dioxide may be estimated to be 7.51 while that of human blood under comparable conditions, but at 37°C., is approximately 7.45. This comparative alkalinity should favor slightly the greater formation of oxyhemoglo-
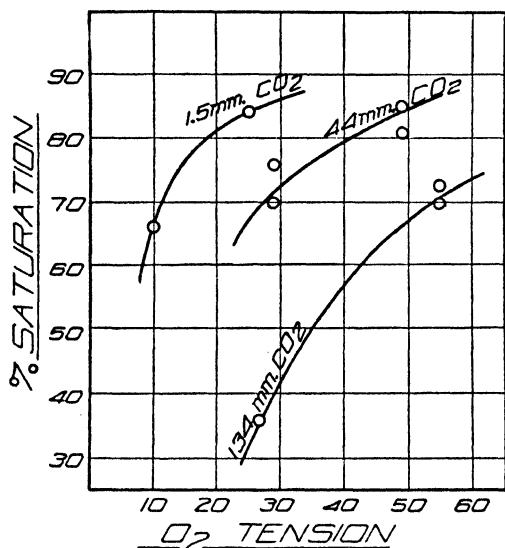
FIG. 6. Showing effect of carbon dioxide tension on the oxygen dissociation curve of the blood of *Pseudemys concinna*. The blood was equilibrated at 25°C. in the presence of the indicated amount of carbon dioxide with oxygen at tensions as shown in mm. along the abscissa. The resulting percentage saturation of the hemoglobin with oxygen is measured along the ordinate.

bin and cause a further divergence in the position of the curves. There is no evidence, however, that these factors have taken effect.

Evidently we are dealing here with a case similar to that described by Krogh and Leitch (19) who find that the affinity of the hemoglobin of the blood of fishes for oxygen is correlated to the respiratory requirements of the species in question rather than to any obvious chemical factors. This difference in the behavior of the blood of the turtle and of man is elucidated by the observations of Macela and

Seliškar (20) which have appeared since the foregoing was written. These authors find that when the hemoglobin of the turtle (and that of a number of other animals) is compared with human hemoglobin, under identical thermal and chemical conditions, the latter has a much greater tendency to combine with oxygen. It would follow from this that the differences which we have observed are due to specific differences in the hemoglobins as well as to such environmental factors as were postulated by Krogh and Leitch.

Effect of Carbon Dioxide.

Fig. 6 shows the results obtained in an experiment to determine the effect of carbon dioxide upon the oxygen dissociation curve. Two points were determined on each curve at 1.5 mm., 44 mm., and 134 mm. carbon dioxide pressures. It is at once evident that the carbon dioxide tension affects the turtle oxygen dissociation curve in the same way that it does human blood (21). Krogh and Leitch (19) have observed the same phenomenon in the blood of certain fish, and Redfield and Hurd (22) have found it to be characteristic of the hemocyanin of the squid.

SUMMARY.

The chief characteristics of the blood of the turtle *Pseudemys concinna*, considered as a system for the transport of oxygen and carbon dioxide, are its low corpuscular content (10 to 22 per cent by volume) and its high concentration of base bound as bicarbonate.

These characteristics account fully for the shape and position of the carbon dioxide dissociation curve, the effect of oxygenation and reduction of the hemoglobin upon the carbon dioxide-combining power of the blood, and the distribution of carbon dioxide between the corpuscles and plasma.

The oxygen-combining capacity of the turtle corpuscles does not differ from that of an equal volume of human erythrocytes. The oxygen dissociation curve is similar to that of mammalian blood and is affected in like manner by the quantity of carbon dioxide present. Its exact shape and position depend in part upon the number of corpuscles in the blood; in part its characteristics cannot be attributed to known chemical factors.

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PHYSIOLOGICAL ONTOGENY.

A. Chicken Embryos.

VII. The Concentration of the Organic Constituents and the Calorific Value as Functions of Age.

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It seems desirable that knowledge of the chemical constitution of the organism at successive stages in the life span be made more precise, so that chemical ontogeny may be correlated with functional differentiation and these data brought into line with the age-old problem of form and function. It is, moreover, of interest to make at least a preliminary analysis of growth into its principal constituent parts so that one may know the extent of growth at least in respect to water, inorganic, and organic substances.

It was with these objects in mind that the experiments included in this communication were conceived. More specifically the study consists of chemical analyses and determinations of the percentage of water, inorganic matter, glycogen, protein, and fat in chicken embryos of 5 to 19 days of incubation age. The chemical findings were checked by calorimetric tests so that the rate at which each separate substance was stored in the body and the total calorific value or potential energy of the embryo could be expressed in terms of age.

Methods.

The embryos, separately or collectively, depending upon their size, were weighed in weighing bottles, cut up into small pieces, and then dried to constant weight in an oven at 102°C. Later, alcohol was added before the embryos were cut up, with the intention of inhibiting enzyme action, but no definite evidence was found that this procedure made any difference in the result. The dried material thus obtained was used for the chemical analyses.

The nitrogen concentration of the dried substance was determined by the Kjeldahl method, and the value so obtained was multiplied by the factor 6.25 to give a rough approximation of the protein content.

The term fat is used to designate the extract obtained after washing the ground-up dried tissue with a mixture of equal parts of alcohol and ether followed by 24 hour extraction with redistilled anhydrous ether in a Soxhlet tube. The material was reground in a mortar with fine sand after the ether-extraction process had been partly completed. The flasks containing the extract were dried to constant weight over paraffin in a vacuated desiccator.

As a rough criterion of the carbohydrate content of the embryo, we analyzed for glycogen with Pflüger's method (1) following closely Cole's modification (2)

TABLE I.

Standardization of Bomb Calorimeter.

	Weight.	Total heat generated.*	Temperature rise.	Water equivalent.	Caloric value when water equivalent is taken as 324.	Error.
	gm.	*cal.*	*°C.*	*cc.*	*cal.*	*per cent*
Cane-sugar..............	1.7723	7050.0	3.032	325	3953.2	0.05
	1.7058	6783.6	2.923	321	3960.8	0.14
Hippuric acid...........	0.6742	3836.7	1.641	338†	5634.1	0.60
	0.9523	5442.9	2.342	324	5668.1	0
	0.8872	5063.8	2.177	326	5663.1	0.09
Average...................................				324		0.2 approximate.

* Total heat generated = weight of substance × caloric value (cane-sugar = 3955.2 cal.; hippuric acid = 5668.2 cal.) + weight of iron wire match × caloric value (1600 cal.).

† This result deviates to an unusual degree from the mean, and was consequently omitted in estimating the water equivalent.

except for the amounts of the solutions used. The final estimations of the glucose formed on hydrolysis were done according to Benedict's quantitative method (2). These analyses for glycogen were made with whole undried embryos. The results expressed in terms of dry weight were calculated on the basis of the values previously obtained for the water content at different ages.

The ash content was determined by careful ignition at dull redness over a Bunsen burner.

Determinations of fuel value were made in a Berthelot-Masler bomb calorimeter as modified by Kroeker.

To obtain the water equivalent for the apparatus, experiments were done with pure samples of cane-sugar and hippuric acid the calorific values of which are known (3955.2 and 5668.2 calories respectively (Table I)).
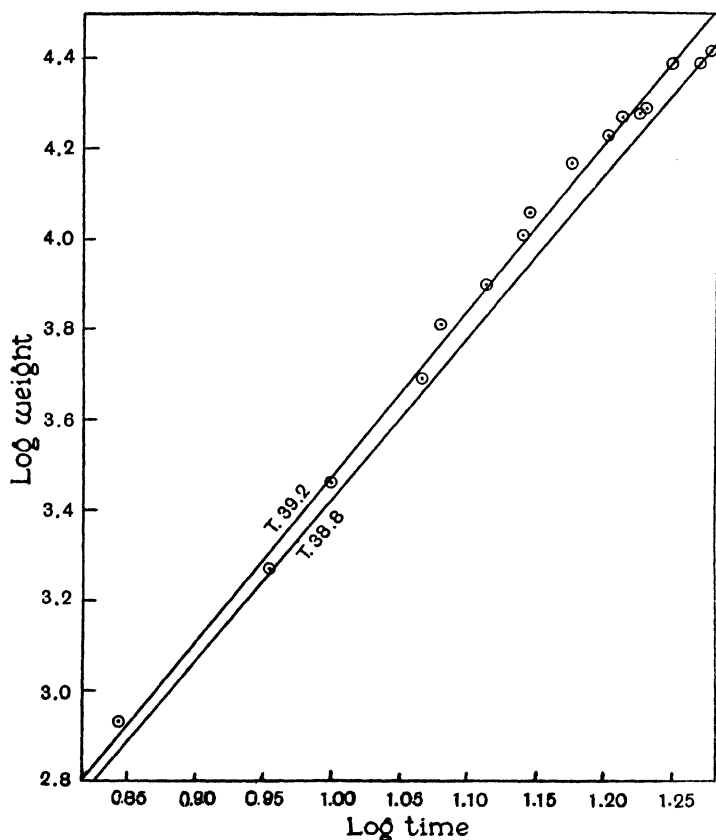
FIG. 1. The logarithm of the weight in mg. plotted against the logarithm of the time. The upper line describes the results obtained in Cambridge (T. 39.2°C.), the lower line the New York results (T. 38.8°)

The Source and Management of the Material.

The eggs used for the estimations of the nitrogen and fat content of embryos were laid during the winter (1923–24) by White Leghorn hens of 8 to 12 months of age kept on a farm in New York State. For these eggs the standards of incubation maintained were as outlined in a previous paper (3) (temperature 38.8°C.; humidity 67.5 per cent). The eggs for the glycogen and calorific value deter-

minations were likewise laid in the winter months (1924–25) by hens of a similar breed and age, but in this case on a farm in the neighborhood of Cambridge, England. The conditions of incubation for these eggs were somewhat different. Instead of a constant temperature room, an electrically heated incubator of Hearson's patent was used. The humidity varied from 60 to 70 per cent, and the temperature from 38.2–40.0°C. with averages of 67.5 per cent and 39.2°C. respectively. The eggs were rolled twice a day at room temperature. For these later (Cambridge) experiments the hens were numbered and trap-nested. It was

TABLE II.

Weights of Chicken Embryos According to Age.

(T. 39.2°C.)

1 Age.	2 No. of embryos weighed.	3 Average weight.	4 Standard error.* ±	5 Log weight in mg.
days		*gm.*		
7	13	0.85	0.03	2.93
9	16	1.86	0.06	3.27
10	15	2.88	0.17	3.96
11.7	4	4.85		3.69
12	10	6.39	0.34	3.81
13	6	7.94	0.48	3.90
13.8	3	10.30	0.19	4.01
14	2	11.36	0.08	4.06
15	5	14.85	0.44	4.17
15.9	4	17.00	0.68	4.23
16.3	3	18.44	0.64	4.27
16.8	2	19.16	0.91	4.28
17	7	19.38	0.48	4.29
17.7	4	24.42	0.67	4.39
18.7	6	24.77	0.52	4.39
19	7	26.59	0.55	4.92

* The standard error $= \dfrac{\text{Standard deviation}}{\sqrt{\text{No. of observations}}}.$

possible by this means to follow the eggs from any one hen, and thus, if necessary, to diminish the extra error entailed by the greater variability of eggs chosen at random from the group.

In the second set of experiments it was noticed that the embryonic weights were somewhat higher and the date of hatching earlier than in the New York series. This difference may have been associated with the shorter period of chilling before incubation or with the slight elevation of the average incubation temperature in the former series. The embryos used at Cambridge may be compared with the much larger series collected during the period of a year and reported in a

previous paper (4) (Fig. 1; Table II). The lower of the two straight lines, corresponding to the formula log W = 3.6 log t − 0.175, was drawn to describe the points obtained when eggs were incubated at 38.8°C. In each case it was found that when the embryo reached a weight of about 25.0 gm. there was a further diminution in growth rate and the subsequent weights fell off the straight line formed when log weight was equated with log time. In the series incubated at 38.8°C., a weight of 25.0 gm. was reached on the 19th day, about 24 hours before the most advanced embryos commenced to hatch. In the later series of eggs incubated at 39.2°C. these events occurred 1 day sooner; that is, the embryos had attained an average weight of approximately 25.0 gm. on the 18th day
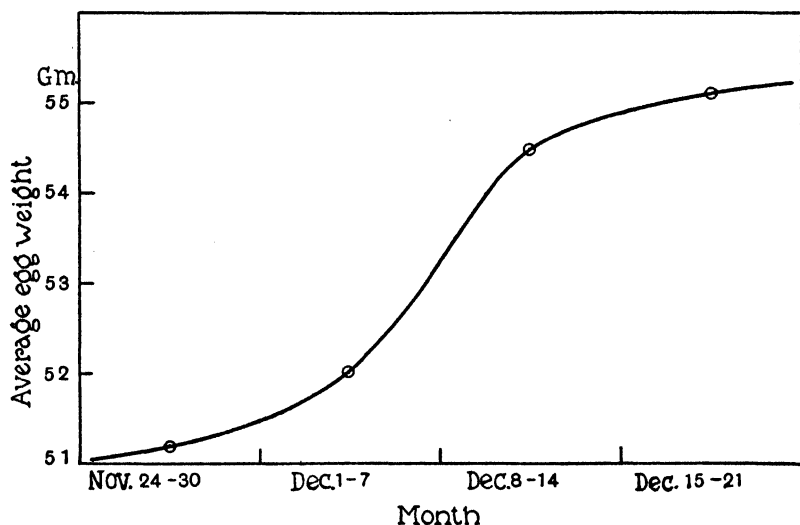
FIG. 2. To show the increase in egg weight during the early weeks after the initiation of laying.

and by the 19th day a number of the chicks had commenced to hatch, and as may be observed the point on the double logarithmic chart had fallen below the straight line. It must be pointed out that it would be a mistake to consider temperature the only factor responsible for the difference in the position and slope of the two lines since other variables were involved.

A study of correlations between the age of the hen, the chemical constitution of its egg, and the subsequent differentiation of the embryo would bear on the problem of aging. For this we have no directly relevant data. Attention is called however, to the accompanying figure (Fig. 2) which shows the change in the weekly average weight of eggs following the initiation of laying by a group of young pullet hens. It is not known in what other respects the eggs differed, but in

The Dry Weight of Chicken Embryos at Successive Ages.

1	2	Solid substance			6	7	8	9	10
		by analysis.		from curve.			Daily increment dry weight.	Mid-increment dry weight.	Growth rate of dry substance.
Age.	No. of embryos.	3 Gm. per 100 gm. H$_2$O.	4 Per cent of total weight.	5 Per cent of total weight.	Wet weight embryo.	Dry weight embryo.			
days					mg.	mg.	mg.	mg.	per cent
5	136	5.62	5.32	5.32	221	11.75			
							11.85		
6	79	5.95	5.61	5.58	423	23.6		15.7	66.5
							19.4		
7	44	6.22	5.85	5.85	735	43.0		25.1	58.4
							30.8		
8	16	6.68	6.26	6.21	1,189	73.8		37.5	50.8
							44.3		
9	37	7.52	6.99	6.50	1,817	118.1		56.2	47.5
							68.2		
10	32	7.34	6.84	7.00	2,661	186.3		85.3	45.7
							102.5		
11				7.70	3,750	288.8		131.6	45.6
							160.7		
12	20	9.25	8.47	8.80	5,105	449.5		200.8	44.7
							241.0		
13	14	11.36	10.20	10.10	6,839	690.5		325.2	47.1
							409.4		
14	16	13.96	12.25	12.25	8,974	1,099		492	44.7
							575		
15	5	18.03	15.28	14.60	11,460	1,674		630	37.6
							686		
16	4	19.18	16.10	16.40	14,390	2,360		708	30.0
							730		
17	19	20.79	17.22	17.22	17,950	3,090		763	24.7
							797		
18	42	21.42	17.64	17.69	22,030	3,887		814	20.9
							832		
19	12	21.12	17.44	17.70	26,670	4,719			

The results in this table are given without their standard errors. The variability of the individuals could not be obtained as the embryos were in most cases analyzed collectively.

.In Column 5 are given the values as read from the smooth curve. These will be used in the calculations involving dry weight.

In Column 6 are listed the figures for chicken embryo weights as given in a previous paper.

Column 7 = Column 5 × Column 6.

Column 8 shows the differences in the successive values given in Column 7.

In Column 9 are given the mid values between successive increments. These figures are meant to approximate the daily increment rate of dry growth.

Column 10 = $\dfrac{\text{Column 9}}{\text{Column 7}}$, or the percentage rate of dry growth.

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view of the possibilities it is considered desirable to record in the future the season of the year and the age of the hens from which eggs used for experimental purposes were derived.
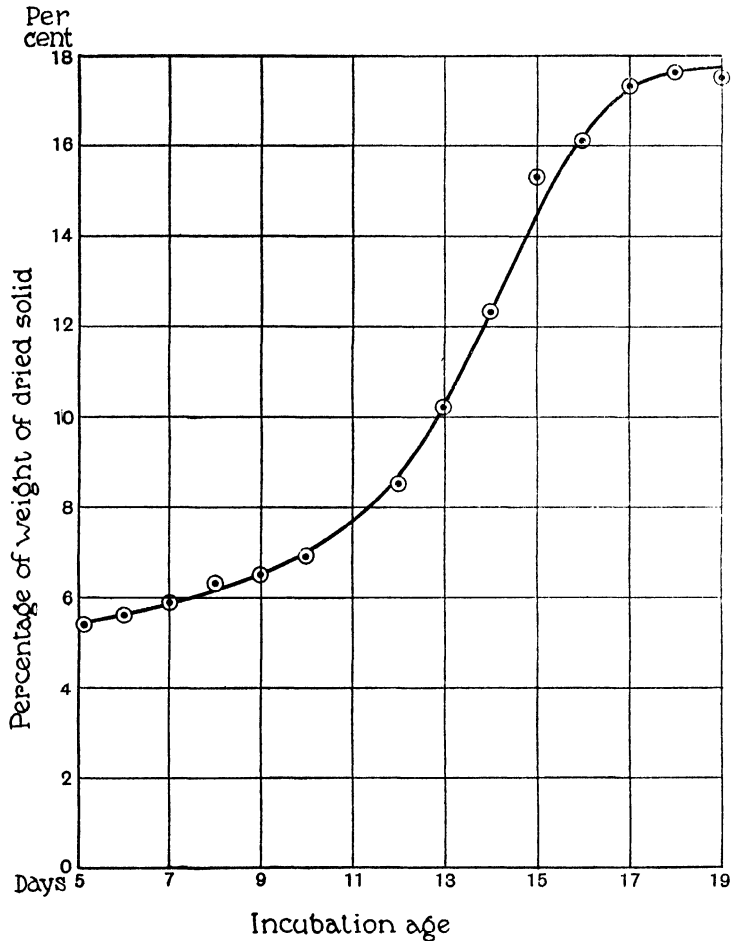
FIG. 3. The percentage by weight of dried tissue as a function of the incubation age of chicken embryos.

RESULTS.

It is apparent that the results of the chemical analyses show considerable variations. For our present purpose it seems that the data

are adequate. Should the necessity arise for more precise measurements they may be supplemented by further observations so as to obtain more representative statistical averages for each age.

The figures for the percentage of dried solid in terms of age are fairly reliable, due to the relatively large number of determinations (Table III). The curve (Fig. 3) shows that the proportion of solid matter to water increases with age, the most marked acceleration of

TABLE IV.

Nitrogen Content of Chicken Embryos As a Function of Age.

Age.	No. of determinations.	Average No. of embryos for each determination.	Nitrogen per 100 gm. of dry substance.	Standard error.* ±	6.25 N or protein per 100 gm. of dry substance.
days			*gm.*		*gm.*
6	8	5	11.1	0.6	69.4
7	10	5	11.2	0.2	70.0
8	4	2	11.6	0.8	72.5
9	1	1	12.2		76.2
10	3	1	11.3	0.1	70.6
11	1	1	11.4		71.2
12	3	1	10.8	0.6	67.5
13	2	1	11.3	0.5	70.6
14	2	1	11.4	0.3	71.3
15	4	1	11.8	0.4	73.8
16	1	1	10.5		65.6
17	2	1	11.1	0.2	69.4
18	2	1	11.2	0.3	70.0
19	4	1	10.0	0.2	62.5
20	2	1	9.4	0.4	58.8

* The standard error $= \dfrac{\text{Standard deviation}}{\sqrt{\text{No. of observations}}}$.

the tendency occurring during the third quarter of the period under inspection. This phenomenon has frequently been observed in numerous different organisms, but recorded in a more or less unsystematic fashion. Tangl (5) pointed out that phylogeny, as well as ontogeny, showed the same tendency towards desiccation with evolution. Aron (6) collected a large amount of data on the subject in 1913, and more recently Moulton (7) has summarized the main facts at hand and has added figures obtained by himself and coworkers from cattle and swine.

None of the available figures give an adequate account of the process during the embryonic period, especially of chicken embryos, and over no period of the life span are they sufficiently precise to be used as a basis for calculation. Although our own figures showed maximum
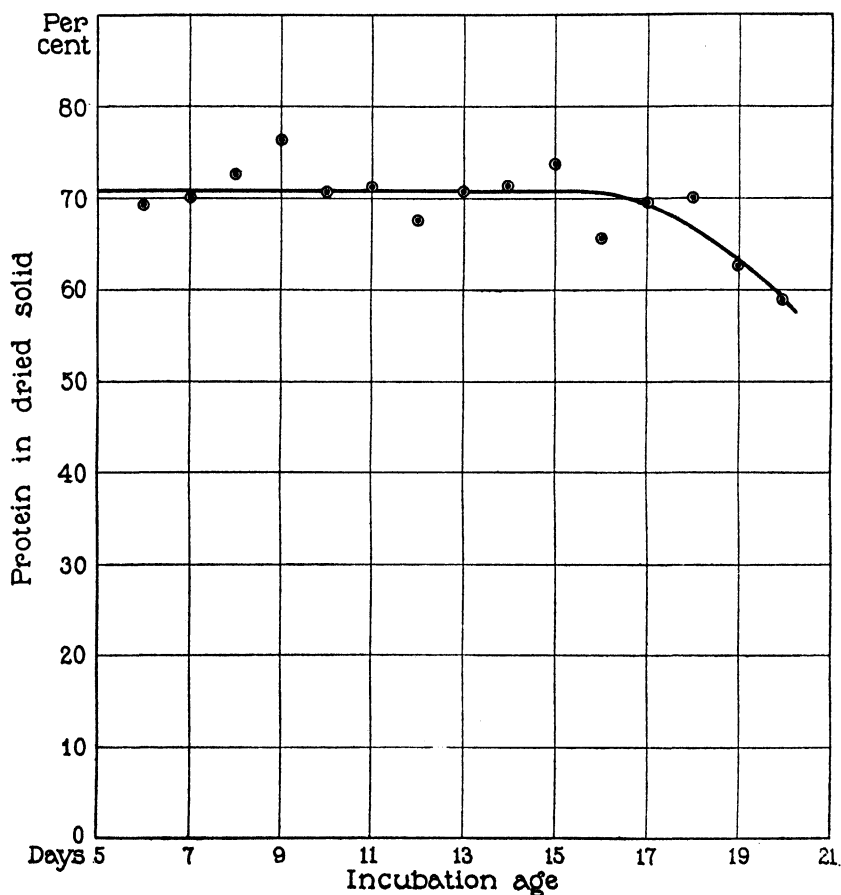
FIG. 4. The percentage of nitrogen ($\times$ 6.25) in the dried tissue of chicken embryos as a function of age.

variations of about $\pm$ 15 per cent about the mean for each age, the number of determinations and the smoothness of the curve suggest that the results have sufficient validity. Due to the importance of

water for all chemical reactions the fact that there is a gradual diminu-
tion in its percentage concentration with age is suggestive of concomi-
tant dissipation of functional capacity.   This notion has formed in
the past a starting point for speculation concerning the necessary
conditions of senescence.

Many other investigators have observed the fact that the nitrogen
and thus presumably the protein content of the tissues increased with
age, but no conclusion was possible as to whether this was the result
of relative water loss or of an actual change in the proportion of the

TABLE V.

Ether Extract (Fat) Content of Chicken Embryos As a Function of Age.

Age.	No. of determinations.	Average No. of embryos for each determination.	Fat per 100 gm. of dry substance.	Standard error.* ±
days			*gm.*	
5	3	35	13.0	0.4
7	1	28	17.3	
9	1	24	17.5	
10	3	10	17.2	0.4
11	1	5	17.5	
13	3	7	19.0	1.5
14	1	2	17.1	
15	1	2	17.9	
16	2	2	18.7	0.3
17	14	1	22.6	0.5
18	7	1	25.4	0.7
19	15	1	28.0	0.7
20	4	1	31.5	0.7

$$* \text{ The standard error } = \frac{\text{Standard deviation}}{\sqrt{\text{No. of observations}}}.$$

protein to the other solid constituents of protoplasm.   Our own figures
are scanty and not entirely conclusive[1] (Table IV).   They may be
represented graphically (Fig. 4).   The curve through the average
points indicates that throughout the greater part of incubation the
concentration of protein in the dried tissue remains approximately 70
per cent.   During the last 3 or 4 days there is a gradual decrease,
which concurs in time, as will be seen later, with an increase in the

[1] The nitrogen determinations were done by Miss Alma Rosenthal and Mrs.
Edith L. Wile.

fat content of the embryo.   It is as if protein were displaced by fat.
The figures obtained by Riddle (8) on the yolk of pigeon eggs give
the reciprocal of this phenomenon.
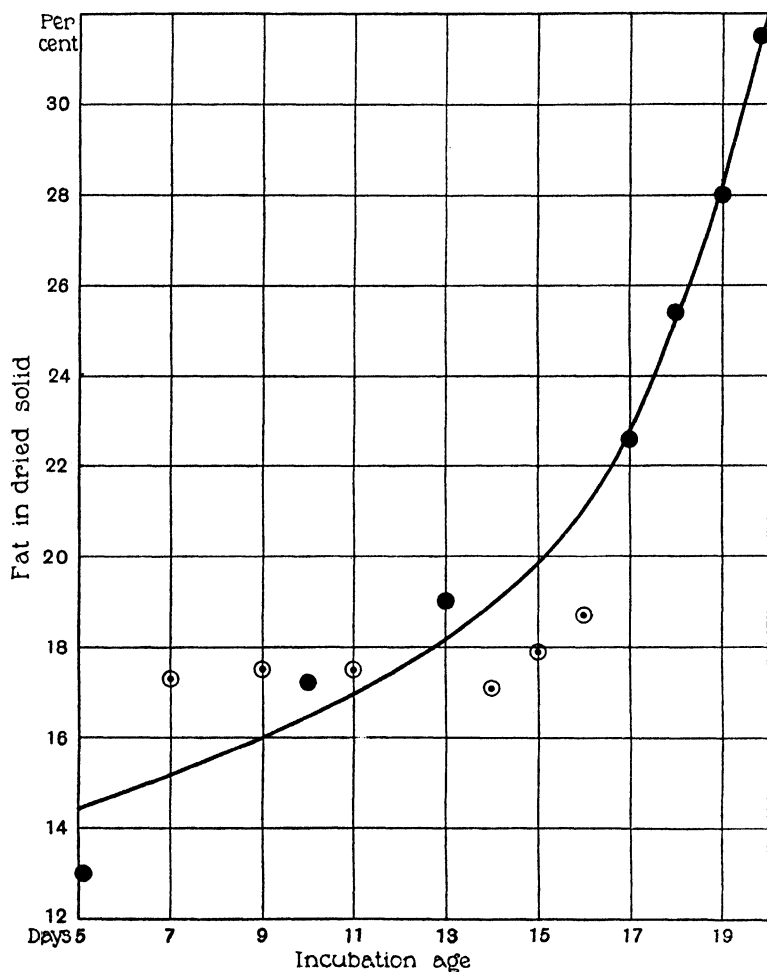
FIG. 5. The percentage of fat (ether extract) in the dried tissue of chicken em-
bryos equated against the incubation age.   The black circles represent the average
of three or more determinations.

The figures for fat show deviations of similar magnitude from the
mean (Table V).   It is perfectly clear, however, from the graph

(Fig. 5) that there is a gradual increase in the fat content with age which is most marked during the fourth quarter of the embryonic period.   It is not at all certain what form the curve may have during the first half of incubation, but this is of minor importance.   During the time of marked positive acceleration a sufficient number of analyses were made to identify within a reasonable degree of accuracy the slope of the line.   Plimmer and Scott (9) have found that beginning with the 16–17th day of incubation there is a marked increase in the ratio of inorganic to organic (ether-soluble) phosphorus, so that it seems probable, in the opinion of these authors, that at this time glycerophosphoric acid compounds become converted into inorganic phosphate

TABLE VI.

Glycogen Content of Chicken Embryos As a Function of Age.

Age.	No. of analyses.	Average No. of embryos for each analysis.	Glycogen in whole embryo.	Standard error. ±	Dry substance in embryo.	Glycogen per 100 gm. of dry substance.
days			*per cent*		*per cent*	*gm.*
5.7	1	40	0.0085		5.5	0.155
11.7	1	8	0.0223		8.5	0.262
13.7	2	3	0.0314	0.0065	11.6	0.271
14.7	3	2	0.0561	0.0070	13.9	0.409
15.7	4	2	0.0556	0.0055	15.9	0.350
16.7	4	1	0.0610	0.0055	17.0	0.359
17.7	2	1	0.0824	0.0001	17.5	0.471
18.7	4	1	0.0732	0.0140	17.7	0.413
20	1	2	0.0753		17.7	0 425

for the calcification of the bones.   This suggests a considerable mobilization of fat, and is further supported by the finding that catabolism during this period is almost solely at the expense of fat.   The theoretical curves to describe the experimental points in the protein and fat charts were drawn freely with special consideration for the values .representing the greatest number of observations, and also with a view of fitting the data from the other analyses.   In other words, they are *possible* curves for the special points in question and *probable* curves when the figures for protein, fat, and ash are considered together.

The analyses for glycogen (Table VI) indicate that with age there is an increase of this substance when expressed as percentage of dried

weight.   At no time, however, does it exceed 0.5 per cent, which is approximately the standard error of the protein and fat determinations.   For this reason glycogen need not be included in the calculations for the calorific value of the solid substance of tissues.   The graph (Fig. 6) shows that glycogen increases most rapidly during the
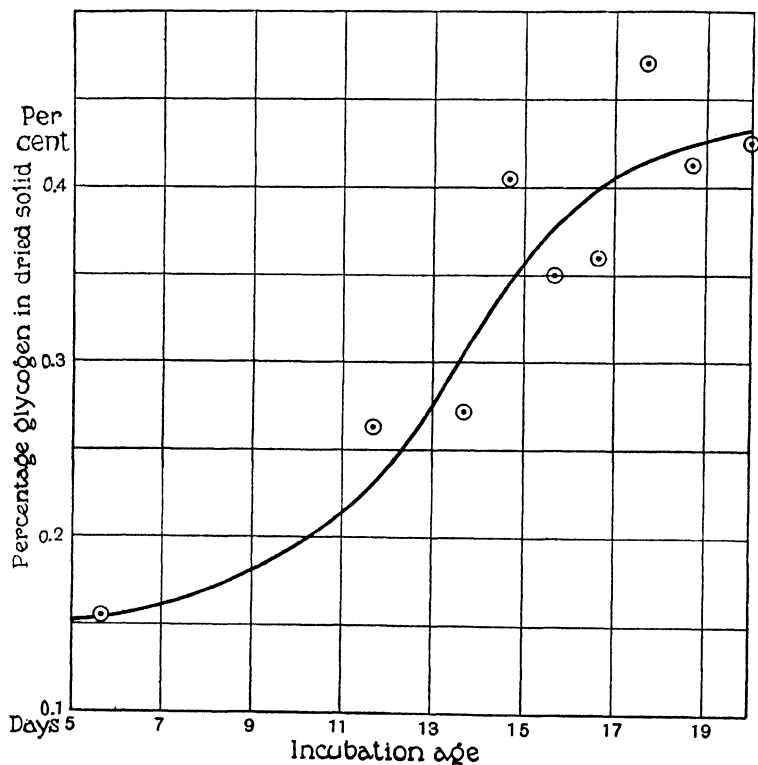
FIG. 6. The percentage of glycogen in the dried tissue of chicken embryos as a function of the incubation age.

second and third quarters of the period under discussion.   Asher (10) has recently thrown some doubt on the accuracy of Pflüger's method for the estimation of this substance.   By the newer method of Rona and van Eweyk (11) he obtained somewhat lower results, and suggests that substances other than glycogen are included in the total obtained by Pflüger's technique.   Among those who have estimated glycogen in the organism there has been considerable disagreement.

Only one more or less systematic study of the embryonic period has been found in the literature. Mendel and Leavenworth (12) reported for the pig embryo values increasing from 0.25 to 0.69 gm. per gm. of wet weight during the latter half of the embryonic cycle. Unfortunately the water content of the tissues was not estimated. Claude Bernard (13) discovered that glycogen was absent from the

TABLE VII.

Ash Content of Embryos in Terms of Age.

Liebermann.[*]			Author.		Theoretical figures from curve.	
1	2	3	4	5	6	7
Embryo weight.	Calculated age.	Ash per 100 gm. of dry substance.	Age.	Ash per 100 gm. of dry substance.	Age.	Ash per 100 gm. of dry substance.
mg.	*days*	*gm.*	*days*	*gm.*	*days*	*gm.*
288	5.4	15.4	10	11.0	5	14.7
1,399	8.4	13.6	10	11.1	6	14.3
2,006	9.2	14.9	12	10.5	7	13.8
2,289	9.6	12.5	13	9.8	8	13.3
8,140	13.7	10.9	13	9.6	9	12.8
12,100	15.2	7.0	13	10.8	10	12.2
16,450	16.6	7.5	14	7.9	11	13.6
16,500	16.6	8.0	14	10.2	12	10.8
18,150	17.1	7.8	15	7.4	13	9.8
18,850	17.2	7.1	16	7.5	14	8.7
19,640	17.4	7.8	19	7.8	15	7.9
20,610	17.7	7.1	19	8.3	16	7.5
21,140	17.8	5.0	19	8.3	17	7.5
22,100	18.0	9.4	20	7.7	18	7.7
640	6.6	13.6	20	8.4	19	8.0
9,358	7.5	9.5			20	8.4
26,198	19.0	11.9				

* For Liebermann's figures see No. 17 in bibliography. Column 2 was calculated from equation $\log W = 3.6 \log t - 0.175$.

liver during the early stages of embryonic life, an observation which Gierke (14) and Lubarsch (15) later confirmed by microchemical tests on tissues. Glycogen it seemed was to be found mostly in muscle and cartilage. Analyses on young chicks at birth were made by Adamoff (16), who found only traces.

Omitting the carbohydrates as negligible compared to the deviations in the individual embryos and the mean errors of the other analyses,

there are at least two ways of checking our results for protein and fat. The sum of the percentage concentrations of the two latter constituents subtracted from 100 per cent should be equal to the concentration of ash; and if the proper values for the calorific value of protein and
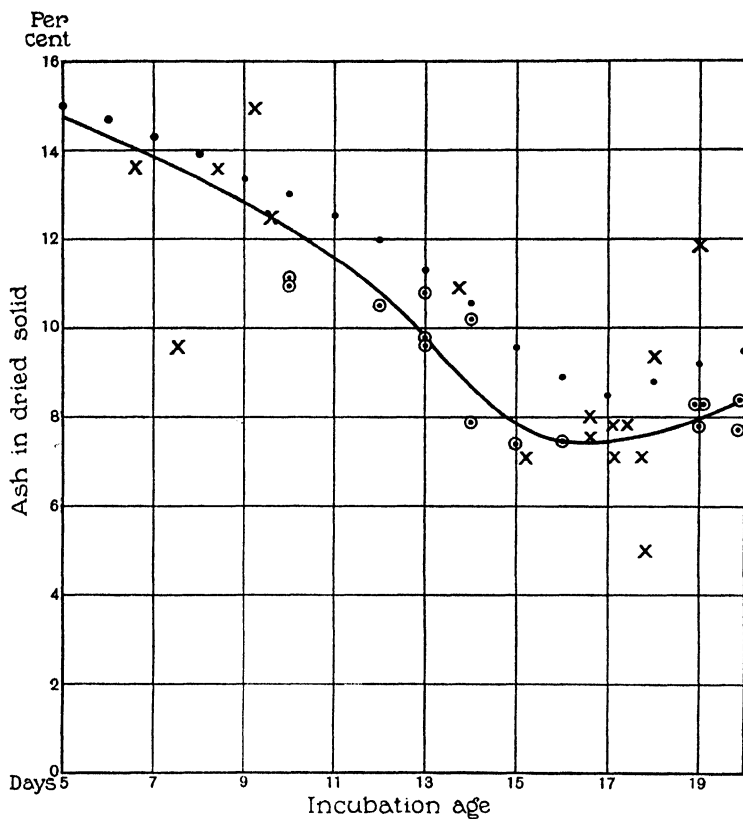
FIG. 7. The percentage of ash in the dried tissue of chicken embryos as a function of the incubation age. X =Liebermann's figures; ☉ =author's results; ● = 100 − (protein +fat).

fat were selected the products of these constants and the results derived by the chemical determinations should agree with the bomb calorimetric findings.

The figures for the ash content of chicken embryos reported by Liebermann (17) were found to conform more or less closely to our own

TABLE VIII.

Calorimetric Determinations of the Fuel Value of the Dry Substance from Chicken Embryos.

Age.	Specimen No.	No. of embryos.	Calories per gm. of dry substance.	Average for each specimen.	Average for each age.
days				*cal. per gm.*	*cal. per gm.*
7	1	17	5.202	5.202	5.202
9	2	21	5.161		
			5.151	5.156	5.156
10	3	15	5.265		
			5.204	5.235	5.235
12	4	10	5.273		
			5.279	5.276	5.276
13	5	6	5.392	5.392	5.392
14	6	1	5.510	5.510	
	7	1	5.371	5.371	
	8	1	5.535	3.535	5.472
15	9	1	5.695		
			5.638	5.666	
	10	2	5.848		
			5.814	5.831	5.748
16	11	1	5.844		
			5.845	5.845	5.845
17	12	1	5.685		
			5.733		
			5.765	5.728	
	13	1	6.077	6.077	
	14	1	6.030		
			6.162	6.096	5.967
19	15	1	5.977		
			5.978	5.978	
	16	1	5.894		
			5.981	5.938	
	17	1	6.260		
			6.221	6.241	
	18	1	6.042		
			6.102	6.072	6.057
20	19	1	6.289		
			6.219		
			6.220	6.242	
	20		6.117	6.109	
			6.100		
	21		6.161		
			6.021	6.091	6.148

(Table VII) and were therefore used to define the curve (Fig. 7) relat-
ing inorganic matter with age. It may be seen that the ash content of
the dried substance is greatest during the early stages of life. Later,
as the time of hatching approaches, the figures suggest a slight rise
which may be due to the precipitation of bone-forming salts at this
period. The small points on the chart are the values obtained when
the combined figures for the percentage concentration of organic
matter (protein and fat) as read from the two curves (Figs. 4 and 5)
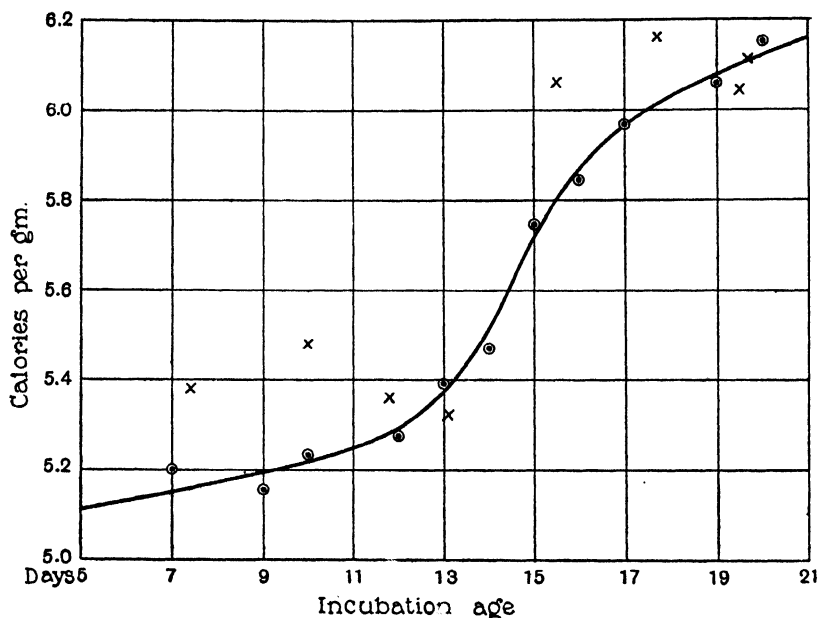
FIG. 8. The calorific value of a gm. of dried tissue as a function of the incubation
age of chicken embryos. X = Tangl's results; ⊙ = author's results.

are subtracted from 100 per cent. (Column 5, Table XI.) Accord-
ing to the curve as drawn there is about 1 per cent of the dried weight
to be accounted for by carbohydrate. This calculation, of course,
is extremely crude, but the closeness of the fit suggests that 6.25
X nitrogen is a fair approximation for the protein concentration, and
that a short ether-alcohol followed by a prolonged anhydrous ether
extraction yields a mixture which may be considered to be mostly fat.

The results of the estimations with the bomb calorimeter (Table

VIII), together with Tangl's results (Table IX) show (Fig. 8) that there is an increase with age in the fuel value of the dried substance of the embryo.   This is evidently due in part to the decrease of the inorganic and increase of the organic fractions of the solid material and in part due to the increasing proportion of fat as compared with

TABLE IX.

Calorific Value of Embryos in Terms of Age.
(After Tangl.)

1 Wet weight of embryo.	2 Calculated age.	3 Dry weight of embryo.	4 Calorific value of embryo.	5 Calories per gm. o dry substance.
gm.	*days*	*gm.*		
0.88	7.4	0.05	0.269	5.38
2.61	10.0	0.19	1.04	5.48
4.71	11.8	0.41	2.20	5.36
6.90	13.1	0.71	3.78	5.32
13.0	15.5	2.57	15.58	6.06
20.8	17.7	3.88	23.89	6.16
24.0	18.4	4.33	27.34	6.32
29.6	19.5	6.07	36.68	6.04
30.2	19.7	5.18	31.63	6.11

TABLE X.

Calorific Value of Unincubated Eggs.

Egg No.	Test No.	Calories per gm. of dry substance.	Average for each egg.
1	1	6922	
	2	6945	6933
2	3	7084	
	4	7083	7084
3	5	6800	6800
Average..			6939

protein.   With age the fuel value of the embryo approaches that of its environment; *i.e.*, yolk + albumin (Table X).

In calculating the heat developed by the oxidation of protein the nitrogen content has been multiplied by 6.25 to obtain the protein content and the result multiplied by 5.7, which is the calorific value of egg albumin and is frequently taken as a representative figure for

tissue proteins. The ether extract content multiplied by 9.3 was used as a measure of the calories from the fat fraction of the oxidized organic material (Table XI). These results may be examined graphically (Fig. 9). The circles represent the calorific value per gm. of organic material calculated on the basis described above, the curve shows the figures empirically determined (*cf.* Fig. 8). It may be seen that there
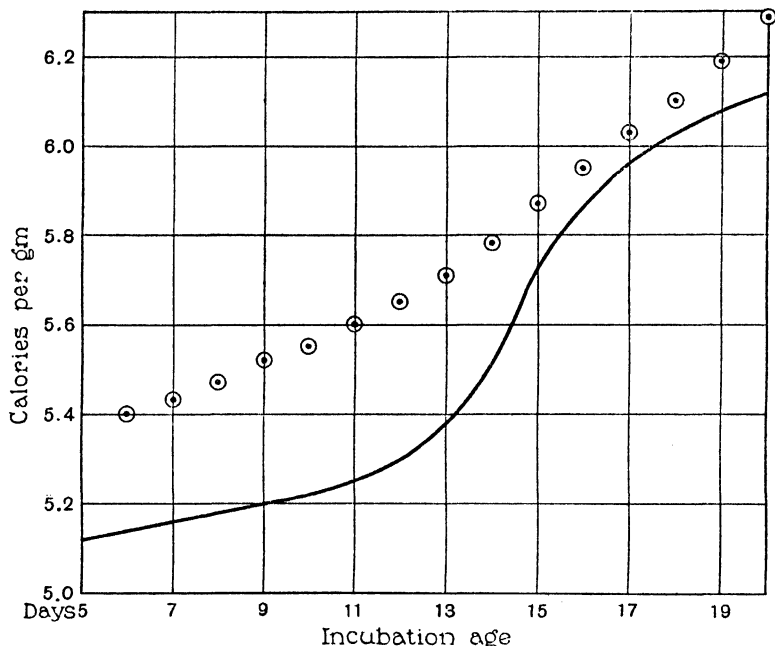
FIG. 9. The calorific value of a gm. of dried tissue as experimentally determined (————) (*cf.* Fig. 8) and as calculated from the nitrogen and fat estimations (⊙).

is a rather wide divergence in the results obtained by the two methods, more marked in the beginning than at the end of the incubation period.

The variability in the results of the actual analytical work may be judged from the figures for the standard errors given in the tables. These are entirely insufficient to account for the discrepancy. It is suggested that either or both of the two constants used in our calculations of fuel value from the protein and ether extract figures, *i.e.*

5.7 and 9.3 respectively, are too high for embryonic constituents during the early days of incubation.   For instance, if one used 5.4 instead of 5.7 for the calorific value of 1 gm. of protein, the figures would be found to agree with the calorimetric determinations.   Moreover, it

TABLE XI.

Figures for the Concentration of Inorganic Matter and the Calorific Value of Chicken Embryos in Terms of Age As Calculated from Nitrogen and Fat Estimations.

1	2	3	4	5	6	7	8
Age.	Protein.	Fat.	Fat + protein (organic matter).	Inorganic matter.	Calories per gm. of dry substance from protein.	Calories per gm. of dry substance from fat.	Calories per gm. of dry substance.
days	*per cent*	*per cent*	*per cent*	*per cent*			
5	70.5	14.5	85.0	15.0	4.02	1.35	5.37
6	70.5	14.8	85.3	14.7	4.02	1.38	5.40
7	70.5	15.2	85.7	14.3	4.02	1.41	5.43
8	70.5	15.6	86.1	13.9	4.02	1.45	5.47
9	70.5	16.1	86.6	13.4	4.02	1.50	5.52
10	70.5	16.5	87.0	13.0	4.02	1.53	5.55
11	70.5	17.0	87.5	12.5	4.02	1.58	5.60
12	70.5	17.5	88.0	12.0	4.02	1.63	5.65
13	70.5	18.2	88.7	11.3	4.02	1.69	5.71
14	70.5	18.9	89.4	10.6	4.02	1.76	5.78
15	70.5	19.9	90.4	9.6	4.02	1.85	5.87
16	70.0	21.1	91.1	8.9	3.99	1.96	5.95
17	68.8	22.7	91.5	8.5	3.92	2.11	6.03
18	66.0	25.2	91.2	8.8	3.76	2.34	6.10
19	62.5	28.3	90.8	9.2	3.56	2.63	6.19
20	59.0	31.5	90.5	9.5	3.36	2.93	6.29

Columns 2 and 3 give the values as read from the curves (figures respectively).
Column 4 = Column 2 + Column 3.
Column 5 = 100 − Column 4.
Column 6 = 5.7 × Column 2.
Column 7 = 9.3 × Column 3.
Column 8 = Column 6 + Column 7.

is clear that the difference between theoretically calculated and experimentally determined results is greater in the early days of embryonic life.   The error involves the use of constants based on the constituents of adult tissue rather than embryonic tissue.   This, if true, would lead to an interesting fact, namely that just as the fuel value per gm.

of organic matter increased with age due to the increased proportion of fat, so also did the fuel value of either or both the protein and fat fractions rise, due to the increasing proportion, within each group, of substances with relatively high calorific values.

The figures for the fuel value may also be used for a further analysis
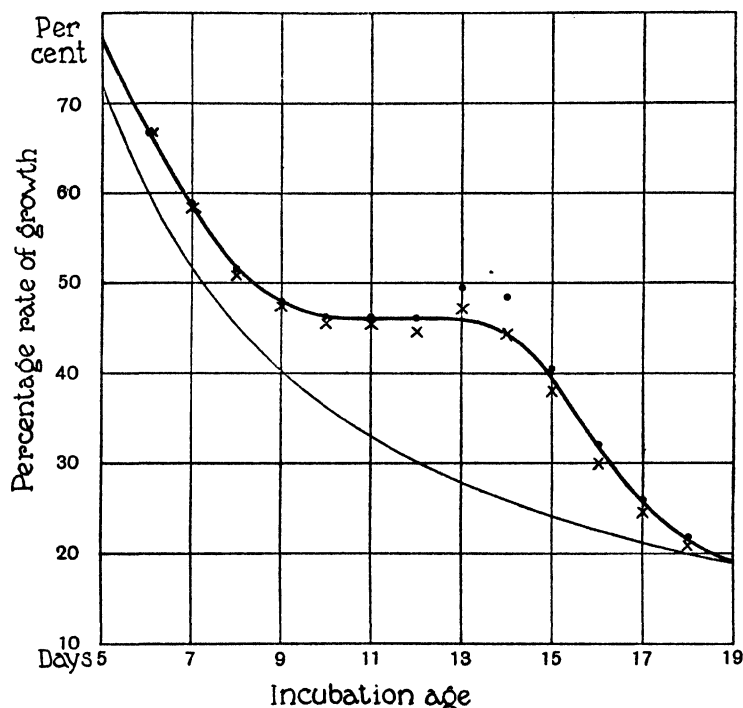
FIG. 10. The percentage rate of growth of dry solid (×) and the percentage rate of growth of calorific value (•), both described by the thick line, compared with the percentage rate of growth of the whole embryo (————) as equated against the incubation age.

of the growth problem. Not only total growth, but independently the rate of growth of the solid substances, of organic matter, or of potential energy may be studied.

In development there are two processes of major importance occurring simultaneously, (1) the integration and (2) the rearrangement of matter. The second to a certain extent may be said to counteract

the first.   If there were no changes in chemical form with age, if for instance the solids "bound" as much water during the latter part of embryonic life as they do at the beginning, the embryo at birth

TABLE XII.

Growth Rate of the Calorific Value of Chicken Embryos at Successive Ages.

1	2	3	4	5	6	7
Age.	Dry weight of embryo.	Calories per gm. of dry substance.	Calorific value of embryo.	Daily increment.	Mid-increment.	Rate of growth of calorific value.
days	*mg.*		*cal.*	*cal.*	*cal.*	*per cent*
5	11.75	5.120	0.0602			
6	23.6	5.135	0.1212	0.0610	0.0807	66.6
7	43.0	5.155	0.2217	0.1005	0.1305	58.8
8	73.8	5.180	0.3823	0.1606	0.1962	51.4
9	118.1	5.200	0.6141	0.2318	0.2951	48.0
10	186.3	5.220	0.9725	0.3584	0.4510	46.4
11	288.8	5.250	1.5162	0.5437	0.7026	46.3
12	449.5	5.290	2.3778	0.8616	1.0976	46.2
13	690.5	5.375	3.7114	1.3336	1.8388	49.5
14	1099	5.510	6.0555	2.3441	2.9361	48.5
15	1674	5.725	9.5836	3.5281	3.8927	40.6
16	2360	5.865	13.841	4.2574	4.4162	31.9
17	3090	5.960	18.416	4.575	4.789	26.0
18	3887	6.025	23.419	5.003	5.137	21.9
19	4719	6.080	28.691	5.272		

The method of calculation used in this table is similar to that employed in estimating the percentage growth rate of dry substance (Table III).

Column 2 is the same as Column 7 (Table III).

Column 3 gives the values as obtained graphically from the smooth curve for the calorific value of dried embryonic tissue (Fig. 8).

Column 4 = Column 2 × Column 3.

Column 5 gives the differences between the values listed in Column 4.

Column 6 gives values which are midway between the increments listed in Column 5, and which tend, therefore, to approximate the instantaneous rate of growth of calorific value for the indicated age.

Column 7 = $\dfrac{\text{Column 6}}{\text{Column 4}}$, or the percentage rate of calorific growth.

would weigh approximately three times as much as it does.   The percentage rate of growth for a dry substance and for the calorific value during the embryonic period may be graphically represented (Fig. 10). The method by which these figures were obtained may be understood

by examining the tables (Tables III and XII). The daily increments of energy for each age are given. As an approximation of the instantaneous rate of energy storage per gm. of embryo the mid values between successive increment values have been divided by the weight of the embryo at that age as obtained by the formula $\log W = 3.6 \log t - 0.175$. The graph shows that, as compared to the whole wet embryo there is during the mid period a temporary cessation of the decline of the growth rate. In other words, the negative acceleration for the growth rate of potential energy is greatest at the beginning and at the end of incubation. The maintenance of a constant rate during the middle of incubation may be accounted for by the great relative increase of solid substance at this period. The values for the rate of growth of the total dry weight follow those for potential energy quite closely, so that the same curve may be drawn to describe them both.

<div align="center">DISCUSSION.</div>

<div align="center">(a)</div>

The organism may be viewed as a locus for the absorption, storage, and elimination of chemical energy. Substances of fuel value which are absorbed may be stored without change of form, may become synthesized and stored as complex constituents of protoplasm, or may be immediately utilized as sources of energy. It would appear that the energy liberated during life is mostly at the expense of the more differentiated chemical elements of protoplasm, glycogen, protein, and fat, and that only rarely are the simpler substances directly burned in the form absorbed; that is, without undergoing a preliminary synthesis.

In view of the uncertainty about such matters the amount of anabolism cannot be estimated. The terms used in the present analysis, however, are subject to direct measurement. It is clear that:

<div align="center">A (absorbed energy) $= S$ (stored energy) $+ E$ (eliminated energy)</div>

When the amounts absorbed and eliminated are equal the organism may be said to be in energy equilibrium and there will be no storage; i.e., the absorption/elimination ratio or more briefly the A/E ratio, will be unity.

In this study we have measured the calorific value or potential energy per gm. of substance at each age during embryonic life. The increments from day to day give an approximate measure of the rate of storage (S).

Since $(A) = (S) + (E)$, we need only measure the daily increment of energy (ΔS) and the amount of catabolism during the same period (ΔE) to know the amount of absorption (ΔA) per day. This matter will be discussed further at a later date when analyses for the respiratory exchange have been completed.

<div style="text-align:center">(<i>b</i>)</div>

Before undertaking these particular experiments we were impressed by what seemed to be a natural scale or gradient, as judged by various criteria, of the chief groups of substances under consideration, namely salt, carbohydrate, protein, and fat,—an order which by similar standards might likewise and with more confidence be made to apply to the substances within each group. It was thought, moreover, that since these organic substances exist in nature as constituents of living matter, progressive changes in molecular complexity would involve greater heterogeneity and thus structural and functional differentiation of the organism. Thus, the elaboration of organic substances and biological evolution might be considered as mutually dependent.

Variations and mutations may be conceived of primarily as new physicochemical states which supply the necessary conditions for new functions; for instance, more complex syntheses; and thus, with time, chemical substances of greater structural and functional complexity would come into being and these in a measure would determine histological and physiological potentialities. It seems, moreover, that with phylogenetic differentiation organisms lose the power to perform some of the simpler syntheses, for which they become dependent upon lower forms; but on the other hand they attain to more complicated chemical activity. Just as one finds in general that along any direct line of descent, as shown for instance by the paleontological record, there has been a gradual increase in the ratio brain mass/muscle mass, so also would change, it is postulated, the ratio fat/carbohydrate. Due to the apparent uniformity of the sequences observed in similar chemical reactions and more generally in all forms of evolution, the

same general tendencies in all direct ancestral progression would be evident whether one assumed monophyletic or pluriphyletic development. Finally with ontogeny, despite one's realization of the limitations of the present modified recapitulation theories, one would expect substances of increasing complexity to be absorbed to form the more differentiated structures, or conversely that the organism, must needs become specially differentiated to utilize for its economy the more complex substances in its environment.

On this basis a tentative prediction was considered, that the following ratios would be found to decrease with age during ontogeny: water/solid; inorganic/organic; carbohydrate/protein; protein/fat. The results reported in the present communication confirm this hypothesis in all respects except one. The carbohydrate, in so far as its concentration may be judged by the amount of glycogen in the tissues, falls out of line. It was found to follow in point of time rather than to precede the protein peak. There may be an error in using glycogen, which is present in only very low concentrations, as index of the total carbohydrate content of the embryo, but, for the present, we must call attention to this exception and let the matter stand. The facts show that the salts are found in greatest abundance at the inception of development, protein during the mid period, and fat at the very end. There are other instances in which these substances may be said to form a similar sequence.

This order holds when carbohydrates, protein, and fat are compared in respect to [1] calorific value and [2] the respiratory quotient resulting from their combustion. It is generally held that the earth has been cooling rhythmically during its history. If so, ever since the first appearance in organic evolution of a mechanism for maintaining a stable internal temperature, there would seem to be an ever increasing need for food substances of greater fuel value. Krogh and Krogh (18) have found that [3] the ratios of carbohydrate/protein and protein/fat in the average diet decrease in general as one proceeds from northern climates towards the equator. The values obtained by Bohr and Hasselbalch (19) for [4] the respiratory quotient during the chick's embryological development suggest a gradual fall with age. Needham (20) has collected evidence to show that glucose is metabolized in the beginning of incubation and it is certain, as many workers

have shown, that fat is burned almost exclusively during the latter stages. As Bohr and Hasselbalch made no correction for the CO_2 absorbed by the yolk and albumin, and therefore retained within the egg, it is probable that their CO_2 values are too low. This correction would affect principally the early part of incubation, since at this time the outside substance (*i.e.* the yolk and albumin) are very great relative to the mass of the embryo. As a result of this omission, probably Bohr and Hasselbalch's figures for the respiratory quotient are too low especially during the initial stages of embryonic life, and the fall with age is actually more pronounced than it is represented. This is another instance of the gradient.

Although there is no general agreement in the matter it is usually maintained that plant preceded animal life in geologic time. The simplest syntheses deriving their energy from the sun seem to involve [5] the formation of the lower members of the carbohydrate series. Certain it is that [6] the carbohydrates are important structural elements and are found most abundantly in plants, whereas fat and protein are more plentiful in animal tissue.

The order of ranking the substances also holds in respect to [7] the site in the gastrointestinal tract of the initial hydrolysis by digestive enzymes and [8] the location, ease, and rapidity of absorption.

Finally a very rough sort of analogy may be called to mind. The experiments of Lyon (21) and Herlant (22) on sea urchin eggs have shown that for a short time after fertilization the interdivisional period is marked by progressive changes in the susceptibility of the cells to various kinds of toxic agents. It was found that in the early stages immediately after cleavage [9] the cells were more affected by water-soluble substances, salts, KCN, acids, and alkalies, and in the later stages preceding mitotic division, by lipoid-soluble agents, chloral, acetone, chloroform, and ether. These findings were attributed to changes in permeability and it was suggested that during the development of each individual cell the constitution of the membrane changed so that at first it was more freely permeable to water-soluble, and later to fat-soluble substances, or in other words the concentration of lipoids on the surface increased with the age of the cell. This cycle for each individual cell seems to have its counterpart in the embryological cycle.

The mere enumeration of these analogies is all that we have to say for the present on the subject of chemical evolution in relation to ontogeny.

SUMMARY.

1. The water, ash, glycogen, nitrogen, and ether extract content of the tissues of chicken embryos were determined between the 5th and the 19th days of incubation.

2. It was found that the concentration of solid substance changed from approximately 5 to 17 per cent during this interval. The chief change in the organic substances involved a relative decrease in the nitrogen as compared with the fat.

3. Bomb calorimetric estimations confirmed these findings. It was shown that the calorific value of the dried substance increased with age.

4. The rate of growth of the total solids, and of the potential energy of the tissues was estimated.

5 Various theoretical considerations were brought forward dealing chiefly with the order in which the chief organic substances, carbohydrate, protein, and fat could be ranged when judged by various criteria.

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SPEED OF TOXIC ACTION OF ARSENIC IN THE SILKWORM.*

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I.

Aquatic animals have been used almost exclusively for investigating quantitatively the toxicity of solutions for metazoans. Terrestrial animals should be more suitable in some respects than aquatic forms since, although larger numbers of the latter can be studied, the manner and locus of introduction of toxic solutions into the former can be varied and controlled; and dying terrestrial animals can be subjected to a wider range of tests and observations to determine the end-points of vital processes and responses to stimuli. Among terrestrial animals certain insects are particularly suitable for quantitative toxicological experiments, since it is possible to obtain large numbers of relatively uniform individuals into which solutions can be precisely introduced by mouth or by injection.

It was found (Campbell, 1926) that several species of phytophagous caterpillars will drink completely drops of neutral arsenical solutions placed in their feeding path. A rather laborious method of feeding known doses of arsenical solutions to individual caterpillars was then worked out, and promising results were obtained.

The present paper describes improved methods and apparatus for toxicological investigations on mandibulate insects, and presents results on the relation of dosage of arsenic to its speed of toxic action in the silkworm.

II.

Among the many mandibulate insects—species of orthoptera, coleoptera, and larval lepidoptera—which were found to imbibe drops of neutral arsenical solutions placed upon the mouth-parts, the silkworm is, perhaps, most suitable for quantitative experiments. It can be reared quickly at any time during the summer in large, highly homogeneous populations. It submits passively to handling, and drinks readily drops of arsenical solutions, which it never regurgitates. Moreover, it is large enough for the application of quantitative subintegumental injections.

A satisfactory test insect having been found, a practicable *per os* method of poison administration depended chiefly on finding an accurate, rapid method of controlling and measuring comparatively minute liquid doses. A micro burette of high sensitivity[1] was designed for this purpose. It merits description, because it should also be useful for micro titrations.

A conventional stop-cock type of micro burette (sensitivity 1 mm.[3]) with side tube for refilling (Billeter and Marfurt, 1923) furnished the point of departure for the writer's model. Rehberg (1925) reached a sensitivity of 0.1 mm.[3] with a mercury pressure type, since he considered it "obviously impossible" to attain that figure with the ordinary stop-cock form. Nevertheless, the writer increased the sensitivity of the stop-cock type to 0.03 mm.[3] by simply reducing the bore of the capillary measuring tube and of the burette tip to suitable dimensions (Fig. 1).

A capillary tube (length, 1 m.; bore, 0.20 mm.) and side tube (length, 1 m.; bore, 5 mm.) were wired upon a meter stick (1 mm. of the scale = 0.0320 mm.[3] by mercury calibration). A rubber tube for mouth suction or pressure was attached to the top of the side tube. The stop-cocks opened into 1 mm. capillary tubes, which were fused to the measuring capillary and side tube respectively. The remaining part (5 mm. tubing) between the tip (bore, 0.08 mm.; outside diameter, 0.14 mm.; length, about 2 cm.) and the capillary of the lower stop-cock was bent at an angle of 45°, and a ground glass joint was interposed in its mid-portion.

With the tip removed and both stop-cocks open, the side tube is filled by gentle suction on the rubber tube while the open glass joint is immersed in the solution to be used. The lower stop-cock is closed, and the liquid rises into the measuring capillary without inclusion of air bubbles at any point. The tip section is separately filled by suction, and is easily slipped into place on the burette without including air.

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[1] A convenient term for the smallest measured volume which may be removed from the tip of a burette.
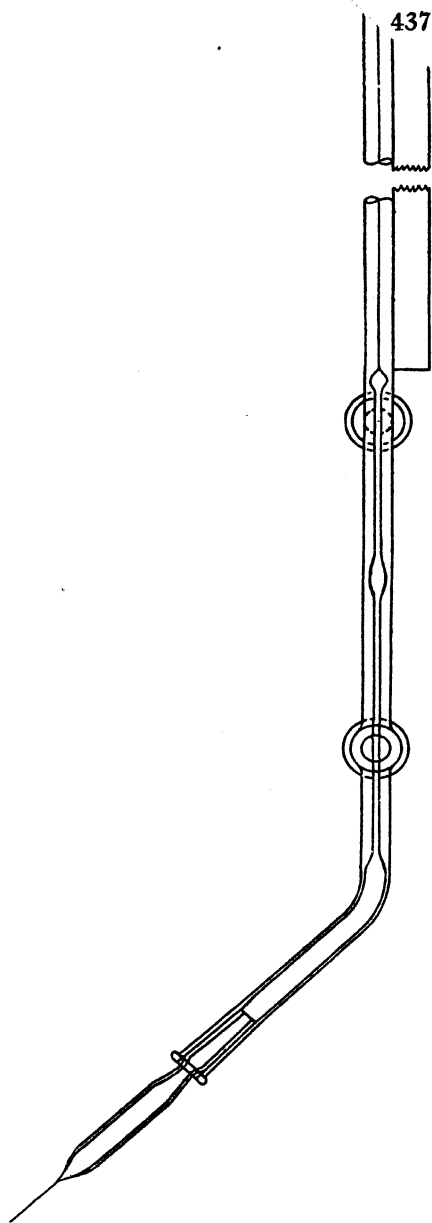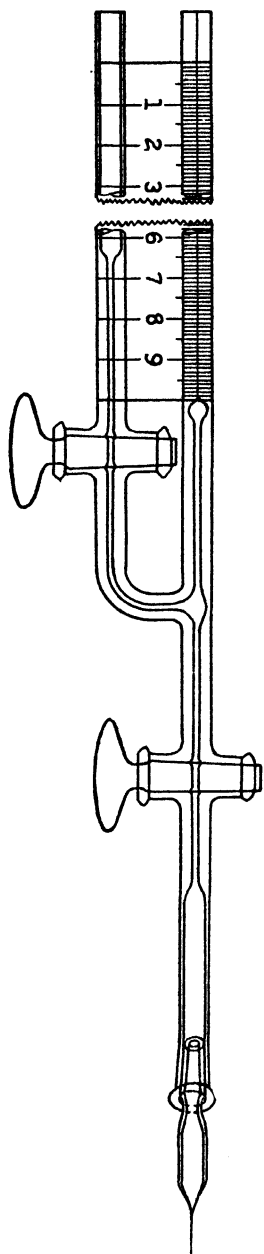
FIG. 1. A micro burette, sensitivity 0.03 mm.³, for measuring minute drops of solutions.

The meter stick provides not only a rigid support for the capillary and side tube, but also a scale background against which the meniscus of the capillary column can be seen distinctly, especially when the source of illumination is behind the operator. The bore of the tip is of such size that it permits a gravity fall of meniscus of about 1 cm. per sec. in the mid-portion of the column when the lower stop-cock is completely open. But the fall of the meniscus can be reduced to a very slow movement by partial opening of the stop-cock, so that the meniscus can be brought to coincide with any desired scale graduation. The stop-cock is handled as little as possible to avoid the slight effect of temperature changes on the position of the meniscus, and when this precaution is taken, it is feasible to estimate its position to 0.1 mm. The outside diameter of the tip is just as important as its bore. It must be fairly uniform over a distance of about 5 mm. from the orifice of the tip, so that a drop will swell out upon it without creeping up the tip. It must be as small as possible so that drops can be removed from the tip without leaving visible residual liquid on the outside of the tip, which is able to hold a drop of water as large as 4 mm.[3] The capacity of the burette is 30 mm.[3]

The burette is emptied by removing the tip, and allowing the liquid in the side tube to flow out. Then the lower stop-cock is closed, and the liquid in the measuring capillary is sucked into the side tube, from which it is allowed to run out as before. The tip section is emptied by thumping the solution out of the joint end, as one shakes down the mercury column of a clinical thermometer.

The weakest point of this instrument lies in the possibility of stop-cock leakage, but if the stop-cocks are well made, and carefully and frequently lubricated, they do not give trouble.

It was necessary to choose between two methods of varying dosage: either one solution could be used, and the dose volume varied; or a number of solutions of different arsenic concentrations could be employed, and a constant volume per gm. of silkworm could be fed. The latter procedure seemed better, since each dose, immediately after imbibition, may then be considered to occupy proportionately the same volume and position in the fore-intestine of every larva, and, therefore, to start on even terms the process of movement along the alimentary tract and of penetration through its epithelium. A constant volume of 5 mm.[3] per gm. was found to be satisfactory. The high sensitivity of the burette made it possible to feed this volume to silkworms weighing as little as 0.010 gm.

A series of neutral sodium arsenate solutions were made up in such a way that the volume to be measured of any solution was numerically equal to the weight of the worm multiplied by a factor, which was set permanently on a slide rule. The poisoning of fifth instar

silkworms (average weight about 2 gm.) was carried out as follows:

A caterpillar in the 2nd or 3rd day of the last instar was weighed to the nearest decigram. The dose volume was calculated by one movement of the glass slide of the rule, and the position on the meter stick to which the meniscus had to be lowered was mentally marked. With the head of the larva, ventral aspect up, between the ends of the thumb and forefinger of the left hand, and with the platinum needle in the right, the lower stop-cock was completely opened, and drops were transferred on the platinum needle from the tip of the burette to the mouth-parts of the caterpillar. Usually it would imbibe the drops as fast as they could be placed upon the mouth-parts. As the meniscus neared its final position, the stop-cock was partially closed, and the meniscus was brought to the previously determined mark; after which the final drop of the poison was transferred to the mouth-parts, and the time was recorded. The residuum of the last poison drop on the mouth-parts was washed into the fore-intestine by a minute drop of water, which was always avidly imbibed. The caterpillar was then placed with a fresh mulberry leaf in a numbered, 1 in. test-tube, open at both ends, in an air bath (temperature, 27.0°C. ± 0.05°). In a similar manner 9 more insects were poisoned. Then the solution in the burette was changed to one of different arsenic concentration, and 10 worms were fed at that concentration; and so on, until an adequate range of dosages had been covered. The sequence of concentrations was chosen in such a way that the operator was free to examine individually all dying cater-pillars.

The top of the air bath was provided with an observation window and with covered arm holes. When caterpillars were seen to have collapsed, they were removed from the tubes, and were stretched, ventral aspect up, on a shelf beneath the observation window. Many preliminary observations on the behavior and responses to stimuli of silkworms dying from arsenical poisoning showed that the failure of the proleg combs to retract on tactile stimulation of the proleg hairs was the most definite arbitrary indicator of the life end-point. It was the last observable response, and failed so sharply that its presence might be noted at one minute, and its absence at the next. Consequently, when this response was about to fail in a series of silk-worms, spread out upon the shelf of the air bath, the operator re-mained over the observation window with arms in the arm holes, and stimulated the proleg hairs of one worm after another, as fast as he could. The response usually failed first in the most anterior pair of prolegs, followed in order posteriorly by the others. The life end-point was taken as the time at which the combs of the last sen-

sitive proleg failed to retract on application of the stimulus just described. The survival times so determined are believed to be not over 2 per cent in error. The relation between dosage of arsenic, administered by mouth, and speed of toxic action (Fig. 2) is discussed in the fourth section of this paper.
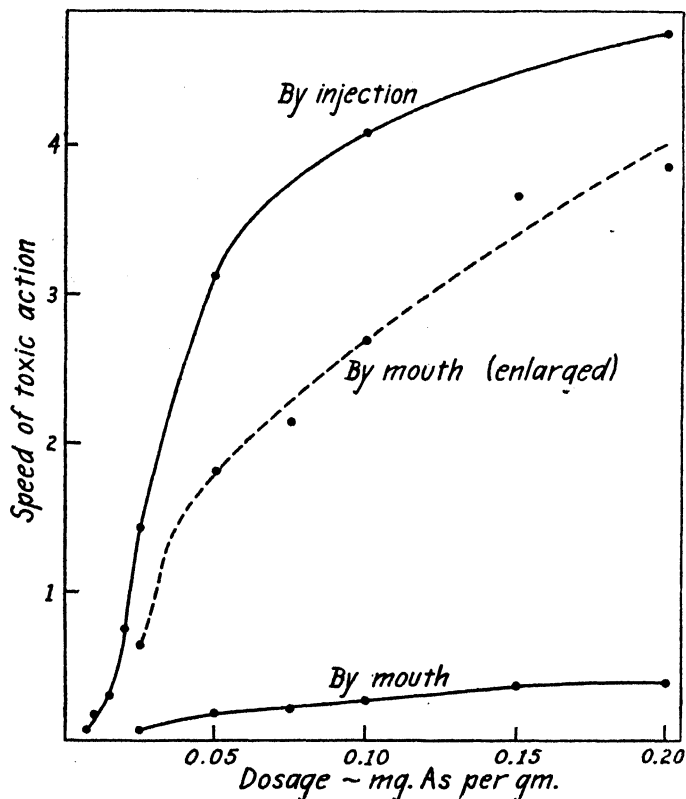
FIG. 2. Speed of toxic action (100 ÷ survival time in min.) is plotted against dosage (mg. As per gm. silkworm). Each point represents the mean of from 5 to 10 determinations of survival time. The probable errors of the mean survival times, except those at the lowest dosage of each curve, are not over 5 per cent of the mean by Peter's formula. The curve "by mouth" is also plotted on a 10 times larger ordinate scale to show the sigmoid shape necessary to give the logarithmic plot of Fig. 4. The minimum lethal dose by injection lies between 0.0050 and 0.0075 mg. As per gm., and by mouth between 0.020 and 0.025 mg. As per gm.

III.

It was thought that quantitative subintegumental injections of the same solutions administered by mouth might provide significant information for interpretation of the *per os* results. Crozier (1922) injected strychnine sulfate solutions into caterpillars with an ordinary hypodermic syringe in a semiquantitative way sufficiently accurate for his purpose. However, quantitative injection of relatively minute volumes of liquid into caterpillars without loss of blood requires a measuring tube and needle of smaller bores than those of any hypodermic syringe on the market. A simple, glass micro-injection pipette met both requirements. The same volume per gm. of silkworm (5 mm.^{3} per gm.) was injected as was administered by mouth. The injections were considered satisfactory only when there was no visible loss of blood. About 9 out of 10 injections were satisfactory. The successfully poisoned silkworms, from 5 to 10 at each dosage, were placed in the air bath, and were treated thereafter exactly like the worms poisoned by mouth. Puncturing the integument and injection of water did not injure the larvæ, since they fed and developed normally after such treatment.

There was no disease nor any other abnormality among the silkworm cultures of these experiments.

IV.

It should be pointed out that the term "speed of toxic action," as used in this paper, does not refer to the time course of destruction by arsenic of, let us say, a respiratory catalyst, but to the relative speed of lethal effect of different dosages, as determined by the reciprocal of the time which elapses from the administration of a dose of arsenic to the disappearance of a definite response in the dying silkworm. The speed of toxic action depends not only on the velocity of chemical reactions leading directly to death, but also on the rate of distribution, excretion, and cell penetration of the arsenic. The shape of the curves relating speed of toxic action and dosage is controlled, therefore, by changes in rate, as dosage varies, among the foregoing processes. It is not to be expected that these changes in rate would occur in such a way that the resultant speed of toxic

action would be directly proportional to dosage over the whole range of possible dosages. The curve "by injection" (Fig. 2), for example, is clearly sigmoid, a type of curve usually found in experiments of this nature. There is probably no direct proportionality between speed of toxic action and dosage in any portion of the curve.

It seems to the writer that the initial acceleration of lethal effect at the lowest dosages may be accounted for by a relatively decreasing rate of arsenic excretion. The final negative acceleration of lethal effect at the highest dosages may be ascribed to the predominance of a relatively declining rate of cell penetration by arsenic which may largely determine survival time. The negative acceleration of cell penetration by organic acids at high concentrations (Crozier, 1916) may be cited to support the possibility of this situation. The limit which the upper end of the curve approaches asymptotically must be partially determined by the time required for the distribution of arsenic, which process is probably independent of dosage.

Powers (1917) and Shackell (1925) have attempted to explain the sigmoid form of curves relating speed of toxic action and dosage. Since neither took into consideration the manifest influence of changes in rate of distribution, excretion, and cell penetration of poison on the resulting speed of toxic action, little need be said here of their theories. Since there are two classes of sigmoid toxicity curves which Shackell did not differentiate, it may be worth while to define them. One class of curves illustrates the percentage of a group of organisms killed as time passes after the administration of the same dose to each individual; the other class, including curves like those of Fig. 2, shows the relation between a series of doses and speed of toxic action. The former class describes variation in individual suscepti- bility to poison, and may be plotted as frequency distribution curves (Fig. 3), if percentage of organisms be changed to number dying during each chosen interval of time (Brooks, 1918–19); the latter class cannot be so plotted, and, therefore, has nothing to do with variation in individual susceptibility, the influence of which is, or should be, eliminated by poisoning a sufficient number of individuals at each dosage.

The data for administration of arsenic by mouth and by injection are plotted to the same scale in Fig. 2 to show the much greater speed

of toxic action following injection. This is to be expected, since a total dose by injection is almost immediately available for carrying out the processes involved in the failure of the proleg response, while only a part of a dose by mouth becomes gradually available on account of the time required for distribution of arsenic along the alimentary tract and for penetration of its walls. Just what portion of
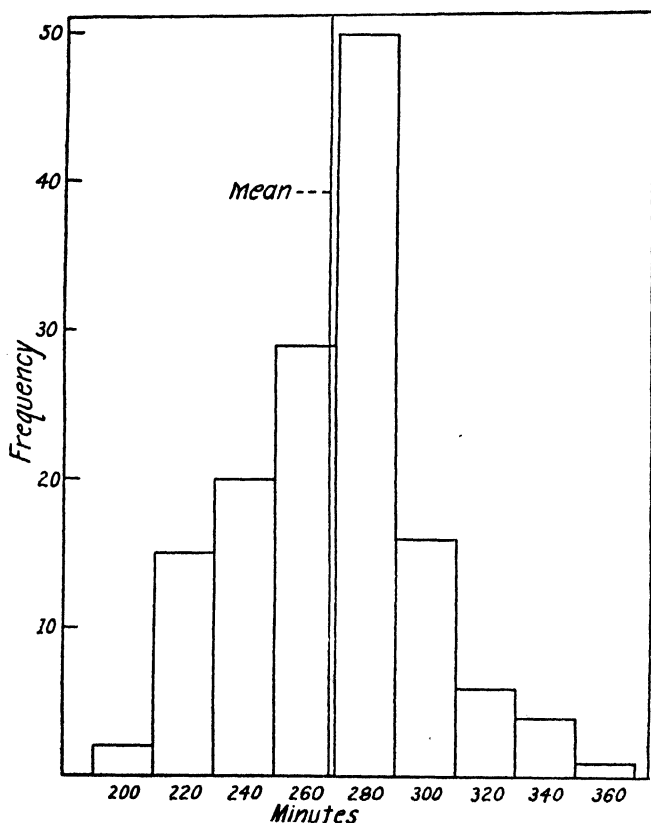
FIG. 3. The frequency distribution of survival times of 143 fourth instar silkworms, fed 0.10 mg. As per gm. The animals were selected to confine weight variation between 0.385 and 0.460 gm. Since there was no correlation between variation in weight and in survival time, the latter may be ascribed to variation in a number of unknown factors, the resultant effect of which is called susceptibility. The chief unknown variable probably was the amount and condition of gut contents when the dose was taken.

a dose by mouth penetrates the gut epithelium cannot be determined from the difference in location of the two curves, because the rates of distribution, penetration, and excretion of arsenic are not known.

The curve "by injection" is fairly well fitted by parts of two parabolæ, and, therefore, when log speed of toxic action is plotted against
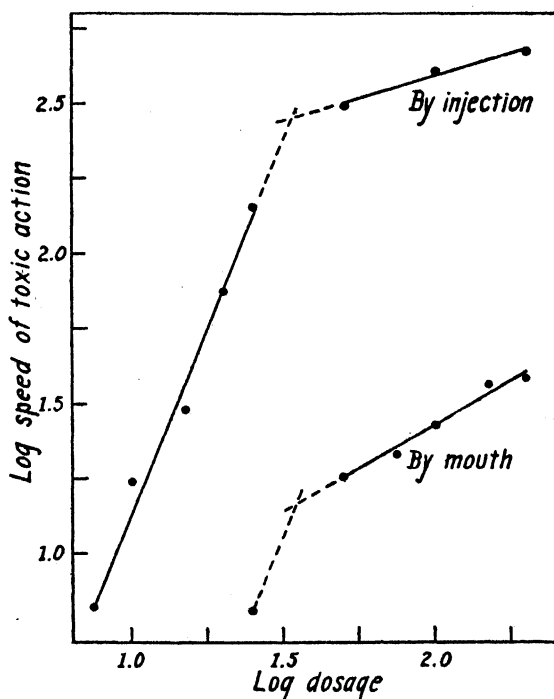
FIG. 4. A logarithmic plot of Fig. 2. At lower dosages the speed of toxic action is proportional to an integral power of the dosage (slope > 1), at higher dosages to a fractional power of the dosage (slope < 1). The lower line for administration of arsenic by mouth is conjectural.

log dosage, the points lie along two straight lines, the lower of slope greater than 1, and the upper of slope less than 1 (Fig. 4). The curve "by mouth" might possibly be fitted in the same way, if observations had been made at the proper dosages. The data of Gueylard and Duval (1922) on the toxicity of four acids to sticklebacks and certain other series of measurements, may be represented simi-

larly by pairs of straight lines. Further experiments are necessary to find out whether it is generally true that speed of toxic action may be proportional to an integral power of the dosage at lower concentrations and to a fractional power of the dosage at higher concentrations, and whether there is an abrupt transition from one relation to the other.

SUMMARY.

A micro burette, micro pipette, and methods for their use in quantitative toxicological investigations on mandibulate insects are described.

It is suggested that the form of curves relating speed of toxic action to dosage may be explained by postulating suitable changes in rate of distribution, excretion, and cell penetration of poison as dosage varies.

The speed of toxic action of pentavalent arsenic in the silkworm is proportional to an integral power of the dosage at lower concentrations, and to a fractional power of the dosage at higher concentrations.

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LIGHT TITRATIONS.

I. The Starch-Iodine Reaction.

By JOHN FIELD, 2ND, AND LOURENS G. M. BAAS-BECKING.

(From the Laboratories of Physiology and Economic Biology, Stanford University, Stanford University.)

Reactions which involve a marked change in color can be measured either with a colorimeter or a spectrophotometer. Both methods have their limitations, the former in the narrow range of color suitable for observation, the latter both in the difficulty of handling and in the subjectiveness of result.

The radiomicrometer, an instrument used by both physicists and astronomers[1] to measure intensities of radiant energy, combines sensitivity, accuracy, and objectivity. It is essentially an Ag-Bi thermocouple with blackened silver receiving surface, connected with a galvanometer. The incident radiation causes a deflection in the galvanometer which is, within wide limits, essentially proportional to the intensity of this radiation. Titrations may be recorded by this instrument (see Fig. 1) in the following manner.

A parallel beam of light, after passing through a hole in an asbestos screen (13) and a cooling vessel (11) is partially absorbed by the titration vessel (10). After passing this vessel it is admitted into the radiomicrometer chamber (14) by the camera shutter and diaphragm (9) and a converging lens (8). The lens converges the rays on an absorbing blackened silver disk (1) which is protected from the outside air by a cover-slip (6), To the silver disk are soldered a very thin silver wire and bismuth wire. Both wires are soldered to a copper coil (2) of approximately the same resistance. The coil is connected with a mirror (3) and the entire apparatus hangs on a quartz suspension (4) which can be changed by a knob (5). The galvanometer magnet (7) is partially omitted in the drawing. The

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[1] Harrison, G. R., *J. Opt. Soc. America*, 1923, vii, 999.

entire apparatus is mounted on a concrete pier. The mirror deflection is read on an illuminated scale by a telescope which swings
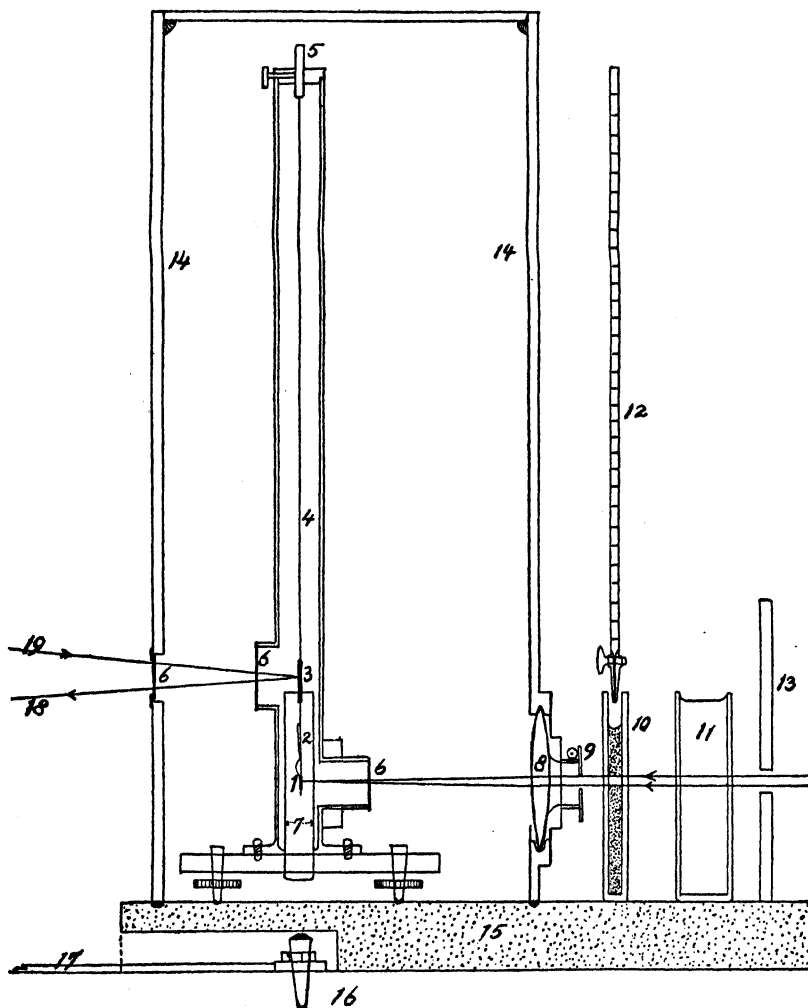
FIG. 1.

on a pendulum (17). The pendulum is pivoted perpendicularly below the mirror suspension (16). As source of light a platinum ribbon filament lamp was run on a transformer. Colored screens

of known spectral transmissivity could be interposed between the lamp and the radiomicrometer.

The instrument was constructed primarily to measure the rates of formation and destruction of biological pigments.

The reliability of the apparatus had to be established by preliminary trials. For this purpose the starch-iodine and ferric chloride-ammonium thiocyanate reactions were studied.

Wheat and potato starches were prepared according to the method of Alsberg and Rask,[2] and ground after the method of Alsberg and Perry.[3] Water-clear solutions were made by sifting the ground starch into rapidly stirred distilled water, and centrifuging the suspension so formed for an hour at a speed of over 2000 R.P.M. In most cases wheat starch preparations are very clear at this point, the supernatant fluid being decanted off and used, but it is usually necessary to filter potato starch solutions several times through kieselguhr to clarify them.

Solutions prepared in this way did not show a very pronounced Tyndall effect, even when exposed to strong illumination.

Definite quantities of starch solutions of known concentrations prepared in this way were placed in a cell made of optical glass (capacity 45 cc.), and standard iodine solution was added from a calibrated sugar burette. The radiomicrometer deflection was ascertained in every case before any iodine was added, and after each addition of iodine. The variability of the lamp was found to be approximately 2 per cent.

After each addition of iodine the solution was stirred, and allowed to stand for 5 minutes before a reading was made. The dark junction of the radiomicrometer was cooled by a fan. The cell was placed as nearly as possible in the same position during a given series of readings, and was carefully washed with distilled water and dried between each run.

It is at once apparent that there are a number of uncontrolled variables in addition to the variation of the Pt ribbon lamp, namely lack of exact similarity of position of the cell in any two runs, slight

[2] Rask, O. S., and Alsberg, C. L., *Cereal Chem.*, 1924, i, 7.

[3] Alsberg, C. L., and Perry, E. E., *Proc. Soc. Exp. Biol. and Med.*, 1924–25, xxii, 60.

temperature variations between series of runs, and the like. However, the total error is not high, as is shown by the data (*cf.* Fig. 2).

The starch-iodine reaction was chosen for investigation by the new method because it is a reaction in which the solution changes from the colorless state to one of pronounced color. Furthermore, there is a threshold concentration of iodine necessary to produce the blue
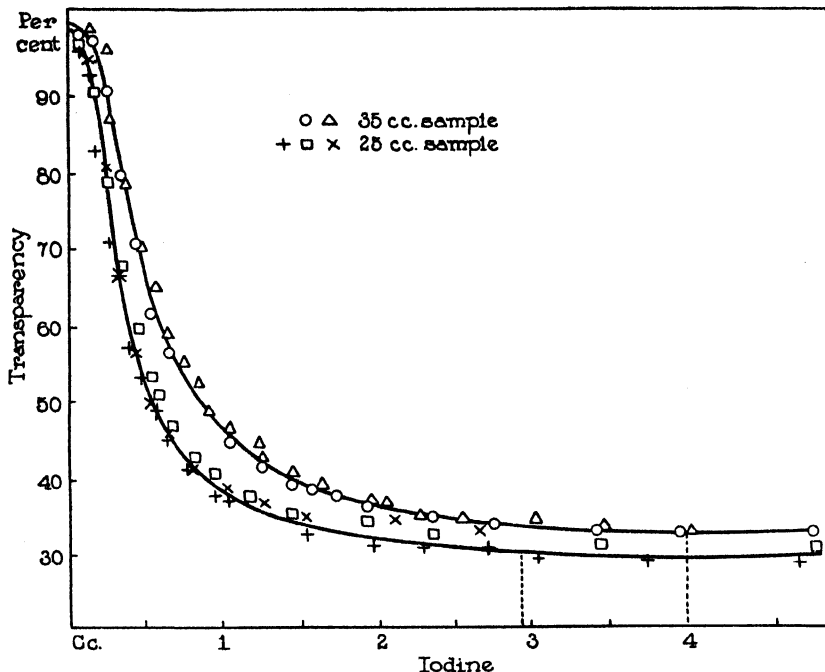
FIG. 2. Relative transparency of potato starch solutions.

color at a given dilution,[4] and it was thought that the precolor stage of the reaction could be followed by the radiomicrometer.

The ferric chloride-ammonium thiocyanate reaction, investigated by Gladstone[5] was considered, but was discarded (at least for the trial runs), since the reaction is so far from complete with respect to formation of ferric thiocyanate at equivalent concentrations of

[4] Meineke, C., *Chem. Ztg.*, 1894, xviii, 157. Treadwell, F. P., and Hall, W. T., Analytical chemistry, New York, 5th edition, 1921, ii, 653.

[5] Gladstone, J. H., *Phil. Tr. Roy. Soc.*, 1855, cxlv, 179.

Fe^{+++} and CNS^-. The starch-iodine reaction, however, goes to approximate completion in a short time.

The accompanying curves show the result of five light titrations with potato starch at room temperature (20°C. ± 1°). Eight titrations were carried out with wheat starch. The ordinates represent transparency in per cent of the original; the abscissæ cc. of iodine (0.8455 mg. per cc.).

Fig. 2 shows the results with two series of 35 cc. samples potato starch (concentration 2.032 mg./cc.). The curve is slightly S-shaped. The end-point, at about 38 per cent transparency, is obtained with about 4 cc. iodine.

Three series made with 25 cc. samples of potato starch of the same concentration are shown on the same figure. Here a transparency of about 31 per cent of the original was reached with a little less than 3.0 cc. of standard iodine. Apparently the amount of iodine taken up by the starch is proportional to the starch concentration ($\frac{35}{4}$ = 8.75; $\frac{25}{2.9}$ = 8.62). The end-point of the curve is not markedly changed by dilution. Transparency as a function of concentration will be considered in another paper. If we assume starch-iodide to be a chemical compound, these measurements indicate an iodine content of approximately 4.5 per cent. Similar curves were obtained with the wheat starch the concentration of which was 0.3278 mg. per cc. 30 cc. samples were used throughout. The end-point is fairly definite at 2.8 cc. iodine, 45 per cent transparency. A starch-iodine compound on this basis contains about 25.32 per cent iodine.

Discussion of Curves.

It was noted, particularly in the case of the wheat starch titration, that the curves did not entirely coincide. However, the points of inflection and end-points are practically the same in every case. The variations in position are to be ascribed to changes in position of the cell, different temperatures on various days, making different gradients between cold and hot junctions etc.

The results seem to justify the conclusion that the apparatus is a suitable one for titration by means of radiant energy. Although the

few observations do not warrant any conclusion concerning the nature of the starch-iodine combination, it may be of interest to remark that our wheat starch-iodide has the highest iodine content on record in the literature.   Our potato starch-iodine combinations on the other hand contain a lower per cent of iodine than any other starch-iodide described, as the following calculations show.

Wheat starch.   Found.   25.32 per cent.

Calculated.   (On $\frac{x}{y} = 2.5$) $= 23.87$ per cent

Potato Starch.   Found.   4.5 per cent

Calculated.   (On $\frac{x}{y} = 17$) $= 4.41$ per cent

The results may be represented by coefficients in the tentative formula $(C_6H_{10}O_5)_x\, I_y$

Starch used.

$\frac{x}{y} = 2.5$	(Field and Baas-Becking).	Wheat.
$= 3$	(Dhar, 1924).	
$= 3$	(Murray, 1925).	
$= 3.3$	(Rouvier, 1897).	Wheat.
$= 4$	(Rouvier, 1897) (Mylius, 1887).	
$= 4.5$	(von Euler and Myrbäch, 1922).	
$= 5.3$	(Rouvier, 1897).	
$= 8$	(Rouvier, 1897).	
$= 9$	(von Euler and Myrbäch, 1922).	
$= 12$	(Anderson and Goettsch, 1902).	Potato.
$= 17$	(Field and Baas-Becking).	Potato.

The value of $\frac{x}{y}$ is apparently at least in part determined by the kind of starch used.

SUMMARY.

1. The usefulness of the radiomicrometer in titration work has been pointed out.   The authors suggest that light titration may also be used where a reaction mixture changes its absorption in the (near) infra-red.

2. The applicability of this method to the starch-iodine reaction has been demonstrated.

THE SALT ERROR OF INDICATORS CAUSED BY STANDARD ALKALINE BUFFERS THEMSELVES.

By JAMES W. McBAIN, OLIVE E. DUBOIS, and KATHLEEN G. HAY.

(From the Department of Physical Chemistry, University of Bristol, Bristol, England.)

A series of well known buffer solutions has been standardised by means of the hydrogen electrode and their true alkalinity thus determined (see, for example, Clark[1]). It is then assumed that the colour of an indicator in one of these buffer solutions corresponds to the true alkalinity of that buffer solution; in other words the colour should be the same as that shown by the indicator in a solution of pure sodium hydroxide of the same alkalinity. We have found that, in many cases, this is not true and it will be shown that very serious discrepancies arise if this unexpected source of error is not taken into account. We have therefore tested the commercially available indicators which are advertised as suitable for use in the range of alkalinity in which we were particularly interested; namely, 0.01 to 0.0001 N. Only two of the simple, and one of the mixed indicators, out of the twenty tested, read correctly at 18°C. This is a higher range of alkalinity than that usually studied by biologists and many of the common indicators are inapplicable. We have carried out no tests on the acid side but it is evident that the same source of error must be looked for there. Indeed Kolthoff[2] found that tetrabromophenol sulphophthalein in phthalate-acid buffer begins to change in colour when pH is 2.8, while in pure hydrochloric acid, the same change begins when pH is 3.5. Since the present work was completed Kolthoff has published a further paper[3] in which he has come to similar conclusions as ourselves, using

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[1] Clark, W. M., The determination of hydrogen ions, Baltimore, 2nd edition, 1922.

[2] Kolthoff, I. M., *Rec. trav. chim.*, 1922, xli, 62.

[3] Kolthoff, I. M., *Rec. trav. chim.*, 1925, xliv, 275.

borate, phosphate, and acetate buffers. The colours shown by indicators in dilute solutions of buffers differed from the colours shown in ordinary, more concentrated buffers of the same pH as measured by the hydrogen electrode. This corroborates our findings by a wholly independent method.

We have also standardised with pure sodium hydroxide the colours given by various indicators at 90°C. Here the modern jargon, which speaks of pH instead of alkalinity is not helpful. With four indicators, (thymol blue, phenol violet, methyl thymol blue (approximately) and *o*-cresol phthalein (approximately)) the so called pH appears the same in cold and in boiling solutions of pure sodium hydroxide, which indicates that the apparent acidity is unaltered; or, in the stricter language of the chemist, the alkalinity has really altered about 100 fold because the dissociation of the water is correspondingly altered. The distorted result is due to the indicator.

With five indicators (phenolphthalein, phenol thymolphthalein, thymol violet, tropeolin O and alizarin yellow G) neither pH nor alkalinity appeared the same at 18° and 90°C. Only three (namely alizarin yellow G, tropeolin O, and thymol violet) have the same colour at 18°C. in pure sodium hydroxide solution and in buffer of identical alkalinity and therefore identical pH. Even with these we have found that the colour is dependent upon the concentration of the indicator. It is a surprising fact that the colour exhibits a constant maximum within a certain concentration of indicator and the colour is diminished if too much or too little indicator is added.

EXPERIMENTAL.

A very simple method of standardisation has been used throughout, namely direct comparison of the colours given by various indicators in standard buffer solutions by matching with the colour produced in dilute solutions of pure sodium hydroxide of known concentration and therefore known alkalinity. In all cases the buffer solution was kept at 18°C. whereas the sodium hydroxide solutions were employed at 18° and also at 90°C. This enables colours obtained at 90°C. to be measured by means of buffers at room temperature and interpreted in terms of true alkalinity. The colour in a solution of pure sodium hydroxide must be taken as normal and the procedure measures

directly the amount of salt error which must be allowed for in the buffer solution itself, since it is found that the colour in the solution of pure alkali is often not the same as that corresponding to the alkali in the buffer as standardised by the hydrogen electrode.

Indicator.	Concentration of solution.	Amount used per 10 cc. solution.
		drops
Alizarin yellow G.	0.01 per cent in water.	10
Universal indicator.		10
o-Cresol phthalein.	0.02 per cent in alcohol.	10
Haematoxylin.	0.2 per cent in 20 per cent alcohol.	5 (0.15 cc.)
2:5 Dinitrohydroquinone.	0.2 per cent in 90 per cent alcohol.	10 (0.2 cc.)
α-Naphthol benzoin.	1.0 per cent in weak aqueous alkali.	5
Thymol blue.	0.04 per cent of mono-sodium salt in 20 per cent alcohol.	10 or 12
or	0.04 per cent in water.	25
Phenolphthalein.	0.1 per cent or 1.0 per cent in alcohol.	Varied.
α-Naphthol phthalein.	0.02 per cent in alcohol.	10
α-Naphthol sulphon phthalein.	0.02 per cent in alcohol.	10
Tropeolin O.	0.02 per cent in water.	10
Brilliant yellow.	0.1 per cent in 20 per cent alcohol.	5 (1.4 cc.)
Methyl thymol blue.		10
Phenol violet.		10
Phenol thymol phthalein.		20
Thymol violet.		3
Iodeosin.		3
Thymol phthalein.		10
Phenol tetrachlorphthalein.		1 (0.06 cc.)
Phenol tetrabrom phthalein.		

If sufficient care is taken to ensure the purity of the aqueous sodium hydroxide there can be no uncertainty as to its true alkalinity. As one check, solutions were observed at 18°C. before and after heating to 90°C.; the colours before and after being always found the same.[4] $N/10$ sodium hydroxide was made up from carbon-dioxide-free drippings from metallic sodium and freshly boiled out conductivity water and the solutions were made in Jena glass. Solutions were prepared immediately before use by dilution with freshly boiled out conductivity water. Hydrochloric acid prepared from constant boiling acid[5] was used to standardise the sodium hydroxide.

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[4] Except in the case of haematoxylin.
[5] Hulett, G. A., and Bonner, W. D., *J. Am. Chem. Soc.*, 1909, xxxi, 390.

Two types of buffer mixture were used, Sörensen and Palitzsch's glycine/sodium hydroxide and borax/boric acid (see Clark[6]). The glycocoll, sodium chloride, borax, and boric acid were Kahlbaum's purest. After addition of indicator buffers were not usually kept in use longer than a day and never longer than 3 days, to avoid error due to fading. Colours were matched by eye, under a Sherringham daylight lamp.

The indicator was added in the amount stated below to 10 cc. of each of the solutions contained in test-tubes of equal bore. Resistance glass was used for the solutions of sodium hydroxide.

Calculation of Results.

Sodium hydroxide is completely dissociated in very dilute solution; the pH value was calculated from the normality

$$pH = \log \frac{1}{H} = \log K_w \Big/ OH$$

using the following values for K_w.

K_w 18°C. = 0.72×10^{-14} (Sörensen).
K_w 20°C. = 0.80×10^{-14} (interpolated).
K_w 90°C. = 53.3×10^{-14} (Lorenz and Böhi).

Direct measurements of the hydrogen exponents of the buffer mixtures were not made and are not required for a relative comparison of the behaviour of various indicators. However, the buffer mixtures were prepared with sufficient care to justify the adoption of the pH values determined by Walbum[7] and Palitzsch.[8] The degree of accuracy attempted was pH 0.2. The results are collected in Tables I and II for the Sörensen standard glycine buffers and in Table III for borate buffers. In the first column is given the pH of the standard buffer as tabulated in Clark[1] and in the second column the concentration of hydroxyl ion that is calculated therefrom; these standard values come from the hydrogen electrode. In the following columns are given the normalities and true pH values of solutions of sodium hydroxide which with various indicators give the same colour as the the standard buffers. If there were no salt error due to the buffers themselves these numbers should all be identical with the values in the first and second columns. It will be noted that in some cases the discrepancies in pH exceed one unit; that is, an error of 1000 per cent.

[6] Clark,[1] pp. 111, 115.
[7] Walbum, in Clark,[1] p. 111.
[8] Palitzsch, in Clark,[1] p. 115.

Results with Glycine Buffer Mixtures.

From the sodium hydroxide values in Table I, or more obviously from the graphical representation of these values, it is evident that the nine indicators investigated fall into two groups.

To one group belong thymol violet, tropeolin O and alizarin yellow G. These indicators show little or no discrepancy between sodium hydroxide pH values and electrometric values for the buffers; that is, these indicators are free from any salt error caused by the constituents of the buffers themselves.

In the second group of indicators the discrepancy is large, that is to say there is a considerable difference in colour between sodium hydroxide solutions and the buffer solutions of similar true pH value. In the buffer solutions the indicator is further transformed to its alkaline colour than in the sodium hydroxide. This means that in measuring an unknown solution of small neutral salt content the observed alkalinity would be too low. Regarding sodium hydroxide as our point of reference, the mean discrepancies in colour amount to 0.79 units of pH for universal indicator; 1.19 pH for thymol blue; 1.0 pH for *o*-cresol phthalein; 0.7 pH for methyl thymol blue; 0.5 for phenol thymol phthalein.

In Table II are given the sodium hydroxide values for 90°C. With the exception of 0.0096 N sodium hydroxide with universal indicator, and the two sodium hydroxide solutions with α-naphthol benzoin, the result of heating the sodium hydroxide is a decrease in the characteristic colour.

In the introduction four indicators were mentioned whose colour is the same in solutions of sodium hydroxide at 90°C. as in glycine buffers of the same numerical pH value at 18°C., although the true alkalinity of the two solutions is very widely different. The following additional five indicators may likewise be used at 90°, again with the proviso that these errors be allowed for since here the sodium hydroxide solutions at 90° which have the same colour as the buffer at 18° have neither pH nor alkalinity in common: phenolphthalein, phenol thymol phthalein,[8] thymol violet,[9] alizarin yellow G, and tropeolin O.

Several indicators not included in the tables may be briefly referred

[9] Colour fades quickly.

TABLE I.

Concentrations and pH Values of Sodium Hydroxide Solutions Giving the Same Colour As Glycine Buffers at 18°C.

Buffer pH = C_{OH}		Thymol violet.	Tropeolin O.	Alizarin yellow G.	Universal indicator.	α-Naphthol benzoin.
pH	C_{OH}					
12.86	0.051	0.046* 12.82	—			
12.67	0.034	—	0.039 12.74			
12.40	0.018	0.027 12.50	0.021 12.47			
12.10	0.0073	0.011 12.18	0.011 12.18	0.0096 12.12		
11.57	0.0027	0.0056 11.89		0.0065 11.96		
11.25	0.0014	—		—	0.0096 12.11	
			Phenol thymol phthalein.			
11.07	0.00085	0.00098 11.13		0.00098 11.13		0.0017 11.22
10.48	0.00022	—	0.010 12.10		—	0.000005 8.86
10.42	0.00020	—	—		0.001 11.11	
10.14	0.000099	0.0001 10.10	0.00033 10.62	Methyl thymol blue.	—	
10.09	0.000098			0.00033 10.62	0.0006 10.87	
9.71	0.000036				—	
9.66	0.000036			o-Cresol phthalein.	0.00025 10.50	Thymol blue.
9.36	0.000016			0.0000997 10.14		0.000197 10.44
8.93	0.000006					0.0000997 10.14
8.58	0.0000028					

*In each case the first number refers to concentration, the second to pH.

TABLE II.

Concentrations of Sodium Hydroxide Solutions at 90°C. Giving the Same Colour As Glycine Buffers at 18°C.

Buffer =		Thymol violet.	Tropeolin O.	Alizarin yellow G.	Universal indicator.	α-Naphthol benzoin.	Phenol thymol phthalein	o-Cresol phthalein.	Phenol phthalein.
pH	C_{OH}								
12.40	0.018	0.046—	—						
12.10	0.0073	—	0.046–						
11.57	0.0027	0.027	0.031	0.0096					
11.31	0.0026	—	0.024	—					
11.25	0.0014	—		—					
11.07	0.00085	0.0096	Metlyl thymol blue.	0.0065	0.0096	0.0017			
10.48	0.00022	0.0056			—	0.000005	0.01		
10.14	0.000099	0.00098	0.01			Phenol violet. 0.00912	0.001		0.0010
10.09	0.0001	—	—		0.0010				
9.71	0.000036	—	—	0.00082	—	0.0039–0.00099		0.000143	0.00072
9.66	0.000036	—		Thymol blue.	0.0007	—		—	—
9.36	0.000016	0.0001	0.00167	0.00066	0.0005	—		0.00082	0.00049
8.93	0.000006	—	0.001	0.00039	—	0.0005		0.00030	0.00030
8.88	0.000006	—	0.00042	—				—	—
8.58	0.0000028	—		0.0001	0.00029		0.00033	0.00020	— —

to. Haematoxylin gave good colours with the buffer solution but changed after about 4 hours; however, the colours obtained with solutions of pure sodium hydroxide (and likewise with soap, or soap and alkali) were unlike the colours in the buffer solutions. The colours obtained with thymol phthalein, α-naphthol phthalein and α-naphthol-sulphon phthalein and especially tetrabromophenol phthalein were transient. The last mentioned indicator pH 8 to 12 like brilliant yellow 8.6 to 9.7, phenol tetrachlorphthalein 8.9 to 9.7, iodeosin 10.1
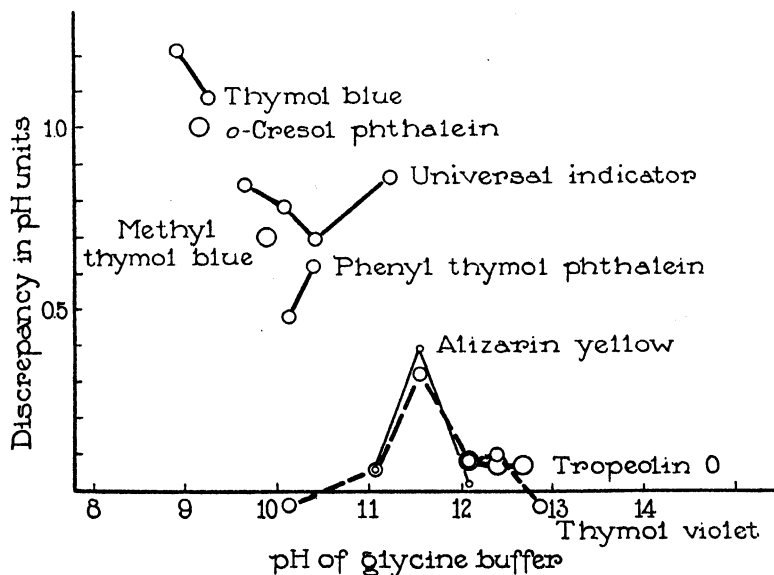
GRAPH 1. The discrepancies between the true pH's of standard glycine buffers (Sörensen) and of solutions of pure alkali which give the same colour with various indicators.

to 12.9, and 2:5 dinitrohydroquinone 8.6 to 12.4, scarcely changed in colour over the range indicated.

Results with Borate Buffers.

All the three indicators investigated show discrepancies of the kind found with glycine at 18°, but here with borate buffers they are far worse: phenolphthalein 1.8 units of pH, thymol blue 1.4 pH, phenol red 2.2 pH (see Graph 2). The buffer solutions are always of a more

alkaline colour than the sodium hydroxide solutions. The error in measuring alkalinity with phenol red exceeds 100 fold if this source of error is neglected.

Phenol red is the only one of the indicators investigated requiring a smaller concentration of sodium hydroxide at 90° than at 18° to produce the same colour.

The ratio of the concentration of sodium hydroxide required at 90° to that required at 18° to produce the same colour with phenolphtha-
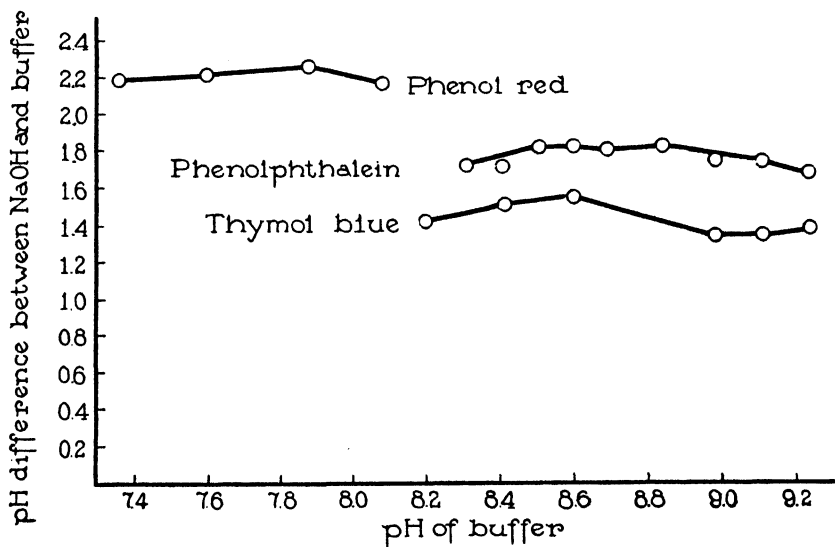
GRAPH 2.   The discrepancies between the true pH's of standard borate buffers (Sörensen) and of solutions of pure alkali which give the same colour with various indicators.

lein varies between individual readings from 1.38 to 2.0 the mean value being 1.5.   The same ratio for thymol blue varies between 1.25 and 3.3 the mean being 2.5.   The two values obtained for the ratio with phenol red were 0.8 and 0.46.

These measurements, even though they do not claim the highest accuracy, do at least show the fact that statements of the "effective ranges" of indicators are incomplete without mention of the specific solution in which they were determined.   For example, phenolphthalein is pale pink in a buffer solution of pH 8.31, but in sodium hydrox-

ide it does not attain the same degree of colour until the pH is 10.13. The data for thymol blue illustrate the same point. With the borate buffers this indicator has just begun to change when the pH value

TABLE III.

Concentrations of Sodium Hydroxide Solutions at 18° and 90°C. Giving the Same Colour As Borate Buffers at 18°C.

Buffer.		Phenol phthalein.		Thymol blue.		Phenol red.	
pH	= C_{OH}	18°C.	90°C.	18°C.	90°C.	18°C.	90°C.
9.24	0.000012	0.00058 10.91	0.00080	0.000296 10.61	—		
9.11	0.0000092	0.00050 10.84	0.00065	0.000197 10.44	0.00061		
8.98	0.0000065	0.00038 10.72	0.00059	0.000148 10.31	0.000489		
8.84	0.0000051	0.00033 10.66	0.00046	—	0.000396		
8.69	0.0000036	0.000285 10.59	0.00038	—	0.00025		
8.60	0.0000029	0.00024 10.52	0.00033	0.000099 10.14	—		
8.51	0.0000023	0.00019 10.42	0.000285	—	—		
8.41	0.0000018	0.00012 10.22	0.00024	0.000058 9.91	0.00012		
8.31	0.0000015	0.000097 10.13	0.00019	—	0.00010		
8.20	0.0000011	—	0.00012	0.000030 9.61	—	—	0.000124
8.08	0.00000087	—	0.000073	—	0.000058	0.000124 10.24	0.000099
7.94	0.0000006					—	0.000062
7.88	0.00000055					0.000099 10.14	0.000046
7.78	0.00000042					—	—
7.60	0.00000029					0.000046 9.80	—
7.36	0.00000016					0.000025 9.54	—

reaches 8.08, which corresponds with the range as usually given pH 8.0 to 9.6. However, at a point near the middle of the colour change, two concentrations of sodium hydroxide were obtained which matched

members of both the borate and glycine series.   Since the colours were
the same, the indicator must have been transformed to the same degree
in all three solutions, and yet their pH values are by no means identical.

pH of Similarly Coloured Solutions at 18°C.

| NaOH | Thymol blue. | |
	Glycine.	Borate.
10.14	8.93	8.60
10.44	9.36	9.11

Kolthoff[10] has placed side by side with the ordinary ranges of the
indicators (presumably in buffer solution) at 18°, the ranges found at
100° in pure dilute acid or alkali.   The apparent shift in range is
therefore due to the sum total of the effects of salt and temperature,
and since these are in opposition to one another the real shift in range
is much greater than that observed by Kolthoff.   For example, his
data for phenolphthalein are:

| Range at 18°. | | Range at 100°. | |
pH	pOH	pH	pOH
8.3–10.0	5.9–4.2	7.9–9.0	4.1–3.2

Our values for the limits of the range determined at both temperatures
in sodium hydroxide solution are (from Table III):

| In NaOH at 18°. $K_w = 0.72 \times 10^{-14}$) | | In NaOH at 90°. ($K_w = 53 \times 10^{-14}$) | |
pH	pOH	pH	pOH
Approximately 9.9 to >11.1	4.3 to <3.1	8.3 to >9.2	4.0 to <3.0

The buffer at 18° corresponding in colour (very pale pink) with these
sodium hydroxide solutions at 90° had pH 8.1 which agrees with
Kolthoff's value 8.3 in the buffer at 18°.   There is also agreement

[10] Kolthoff, I. M., *Rec. trav. chim.*, 1921, xl, 775.

between Kolthoff's value of pH 7.9 at 100° and our value of pH 8.3 at 90°, when the difference of 10° in temperature is taken into account, since increase of temperature decreases the pH.   There is, however, a discrepancy of pH 1.6 between our result for sodium hydroxide at 18° and that of Kolthoff, presumably due to salt error of the buffer used by the latter at 18°.   Incidentally, the values found in sodium hydroxide solution show a large change in pH and only a small change in pOH which is at variance with Kolthoff's suggestion that "Les indicateurs qui sont eux-mêmes des acides faibles sont presque tout aussi sensibles aux ions hydrogène à température plus élevée qu'à la température ordinaire.   Ceux qui sont des bases faibles deviennent moins sensibles aux ions hydrogène mais gardent à peu pres la même sensibilité aux ions hydroxyle."   Phenolphthalein was regarded by Kolthoff as being in accordance with this since the pH range as he recorded it did not change much with rise in temperature, but this was due to his comparing the colour with buffer at 18° instead of with dilute alkali.

For two other indicators, thymol blue and alizarin yellow G it is possible from our data to get some idea of the change of the lower limit of the range in pure sodium hydroxide at 90°.

Thymol blue (in borate buffer pH 8.08).			
NaOH at 18°.		NaOH at 90°.	
pH 9.5	pOH 4.7	pH 8.0	pOH 4.3
Alizarin yellow G (in glycine buffer pH 11.07)			
NaOH at 18°.		NaOH at 90°.	
pH 11.1	pOH 3.1	pH 10.1	pOH 2.2

These two indicators are not in Kolthoff's table; they show a large change in both pH and pOH with change in temperature.

The changes in apparent pH as shown by indicators at 70° were found by Kolthoff by heating buffer solutions whose temperature co-efficients had previously been determined by hydrogen electrode (Walbum).   The method used by the authors, namely that of heating sodium hydroxide solutions and comparing with constant buffers at

18° standardised against the dilute sodium hydroxide at 18° gives values for the changes in apparent pH as shown by the indicators at 90° which are one or two complete units of pH greater than Kolthoff's values; 0.5 of this is due to the change in K_w between 70° and 90°.

Effect of Varying Concentrations of Indicator.

The most satisfactory concentration of indicator is the smallest which will produce a maximum colour in the solution, since at this
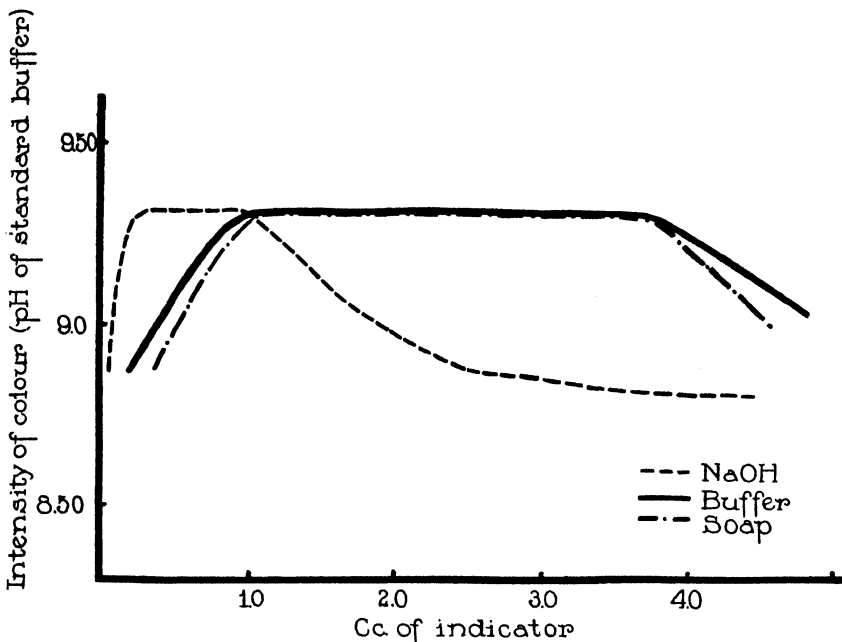
GRAPH 3.   Diagram showing that there is a range of concentration of indicator producing maximum colour which falls off again if too much indicator is added.

point only is the full alkalinity of the solution indicated.   It is, however, important that different amounts of indicator are necessary for the attainment of maximum colour in unlike solutions.   Graph 3 illustrates this fact.   Consider the three solutions sodium hydroxide, glycine buffer pH 9.31, and a soap solution, which all eventually come to the same maximum colour.   Using 0.1 per cent phenolphthalein it was found that 0.25 cc. of indicator was required to produce maximum

colour in 10 cc. of sodium hydroxide solution (0.00026 N). Between 0.9 and 1.0 cc. was required to bring the buffer solution to the same colour.

Experiments with soap solutions were made with another indicator, *o*-cresol phthalein 0.02 per cent. These showed that the soap solution requires the same concentration of indicator as the buffer. The gradual appearance of colour, accompanying a gradual increase in the indicator concentration, was followed by comparing the sodium hydroxide, buffer, or soap solutions, in turn, with a series of buffer standards in which the maximum colour was developed. It was found that the colour of the soap solution increases more rapidly than that of the buffer until the maximum intensity is reached when the two colours become identical.

TABLE IV.

Changes in Apparent pH As Shown by Indicators As Found by Kolthoff by Heating Buffer Solutions to 70°, and As Found by the Authors on Heating in Dilute Hydroxide Only, from 18° to 90°.

Indicator.	Kolthoff.	Authors.
Phenolphthalein.........................	−0.4 to 1.0	−1.6 to 1.7
Phenol red.............................	−0.3	−2.0 to 2.2
Thymol blue............................	−0.35 to 0.45	−1.3 to 1.5

An excess of indicator causes the colours to fade, that of the soap decreasing more rapidly than that of the buffer. On the other hand, comparing the rate of appearance of colour in sodium hydroxide and buffer solutions, it is seen that the sodium hydroxide reaches its maximum colour first, when the concentration of indicator is 0.25 cc. When 0.9 to 1.0 cc. has been added to the buffer the maximum colour is attained, but if 1.0 cc. is added to the sodium hydroxide the colour has already begun to fade. Hence in our further work we have found it advisable to use maximum colours. By using this precaution and availing ourselves of the standardisations described in the present paper we have found several indicators which give values for the hydrolysis of soap solutions at 90°C. in agreement with previous values determined in this laboratory by such methods as hydrogen electrode and rate of catalysis. The results will form the subject of a separate

communication, but it may be mentioned that the universal indicator gives quite misleading results when used with soap solutions.

SUMMARY.

1. It is found that with many indicators there is a big discrepancy between the true alkalinities or pH values of solutions of pure sodium hydroxide and of standard alkaline buffers which give the same actual colour.   This discrepancy must be ascribed to salt error caused by the buffer itself and exceeds in the most extreme case two whole units of pH; that is, an error of 100 fold in determining alkalinity.   In only three cases, namely alizarin yellow G, tropeolin O, and thymol violet, was this error inappreciable.   Most of the thirty indicators tested were found for various reasons unsatisfactory in the alkaline range studied.

2. Several indicators show a maximum depth of colour after sufficient indicator has been added, but above a certain concentration further addition of indicator diminishes the colour again.

TEMPERATURE CHARACTERISTICS FOR PREPUPAL DEVELOPMENT IN DROSOPHILA MELANOGASTER.*

By CHESTER I. BLISS.

(From the Zoological Laboratory, Columbia University, New York.)

Recently attempts have been made to interpret the effect of temperature upon the rate of life phenomena in terms of chemical processes. There are peculiar difficulties, however, in applying such interpretations to development, principally the errors introduced by using an average rate as if it were an instantaneous velocity. This assumes that a single rate-controlling chemical process extends throughout the stage delimited by the markers which time its beginning and end. In one case in which this has been analyzed, the embryonic development of the grape leaf-hopper, a series of successive processes, differing markedly in temperature coefficient, limits the developmental rate (Bliss, 1926). This analysis seemed to warrant further trial under more favorable experimental conditions.

The *Drosophila* pupa which had already been studied by Loeb and Northrop (1917) was chosen as suitable material for testing variations in the temperature characteristic through development and the conclusions therefrom upon the controlling chemical reactions, if chemical. There proved to be several difficulties, however, in using the entire puparial period for the investigation.

Although equal numbers of larvæ formed puparia every hour throughout the day, the adults from these puparia did not similarly emerge in equal numbers.

From about 5 or 6 p.m. until 4 o'clock the next morning in one experiment, for example, the number of adults emerging each hour was less than one-fourth the number of larvæ forming puparia. Beginning at 4 a.m., however, adult flies began to appear in large numbers, and for several hours two to three times as many adults emerged as puparia were formed, this increased emergence continuing until nearly noon. The diurnal fluctuation has appeared regularly, even under constant temperature, light, and humidity conditions. If the minimum time for the stage is used as standard, here 84 to 85 hours at 28.5°C. (female), nearly 75 per cent of the flies did not emerge until from 1 to 9 hours after they had presumably completed development. Accordingly it proved impracticable to use emergence of the adult as an indicator of development.

The puparia, which are more or less transparent, were watched for morphological changes in order to secure markers free from rhythmic variations. The following stages were selected: puparium formation, pupation, first appearance of eye color, change in eye color from orange to red (or first appearance of head bristles which was nearly synchronous at the temperatures used), and emergence of the adult. These stages divided the puparial span into four parts, the first comprising the prepupal period, the last three the pupal stage. The length of each of these stages was then correlated with that of every other one, and the partial correlation coefficients determined. Using the conventional terminology (Yule, 1922), at 30°C.

$$r_{23.14} = -.41\pm.05 \qquad\qquad r_{14.23} = .19\pm.06$$
$$r_{34.12} = -.35\pm.05 \qquad\qquad r_{13.24} = -.13\pm.06$$
$$r_{12.34} = -.31\pm.06 \qquad\qquad r_{24.13} = .02\pm.06$$

Variations in the length of the successive stages should not influence each other if the markers separated them adequately, but the above results show a marked mutual influence between successive stages. The first appearance of eye color seems the most unreliable marker, followed by change in eye color and pupation. This particular experiment was free from the systematic error in time of emergence. On the basis of these data we cannot judge to what extent the variations in the markers were due to relative independence of the visible change from the rate-controlling developmental systems, and to what extent to experimental errors in recognizing the markers—the order of unreliability parallels that of experimental difficulty. Because of the theoretical and experimental advantages of treating each stage independently, the prepupal period alone will be considered here.

Material and Methods.

A mutant race (sooty) of *Drosophila melanogaster*, inbred by brother-sister matings for over twenty generations, insured relative genetic uniformity. The flies were raised on the usual banana agar in ½ pint milk bottles but without paper. When the larvæ reached maturity, they crawled up the sides of the

bottles and formed puparia on the glass from which they were removed at ½ hour intervals with fine, flexible eye-knives. Under such conditions, two workers could examine 30 to 40 bottles and remove the puparia, usually 25 to 30 of them, in less than 10 minutes time. Small black paper blocks filled with 2 per cent agar-agar, provided a moist dark substratum for the prepupæ and could be easily handled in Petri dishes in the incubators. After an appropriate interval these were examined every ½ hour to determine time of pupation. Following pupation, they were raised at a temperature of 25–28° until the sex combs appeared and the sex of the pupæ could be determined.

The temperature was controlled by means of toluol-mercury regulators. Although the thermoregulation was not as reliable as could be desired, in the experiments included here it probably varied within a range of less than 0.3°C. Thermograph records gave practically straight lines. Each temperature was read several times on a mercury thermometer. Except one graduated to ½°, the thermometers were graduated to single degrees centigrade, and all were calibrated at the beginning of the experiment with respect to an instrument that had been checked at the Bureau of Standards.

Larvæ which had everted the anterior spiracles and did not crawl upon handling were deemed puparia. Individuals in which the conspicuous, longitudinal, dorsal tracheæ did not extend unbroken from the anterior to the posterior spiracles, or in which a space had appeared between the posterior dorsal surface of the body and the puparium were judged to have completed the prepupal stage. Both of these markers were sharp and unmistakable.

Morphological Changes in the Prepupal Stage.

When the larva has ceased feeding, it crawls out of the food up the sides of the bottle, and, after approximately 2 hours time, it becomes quiescent, unless disturbed, and everts the anterior horns. The larval skin shortens, loses all signs of segmentation, and becomes the puparium from which the adult eventually emerges. This entire period may be called the puparial stage, and is equivalent to the pupal period of *Drosophila* literature. As first formed the puparium certainly does not contain the pupa as the term is generally understood among entomologists, a fact recently emphasized by Snodgrass (1924). The body completely fills the puparium, the larval longitudinal dorsal tracheæ, heart, and fat bodies are very apparent, and there is no external trace of adult structures. It is not until over 11 hours later (at 25°C.) that pupation occurs within the puparium.

Pupation is begun by a contraction of the body away from the posterior-dorsal region of the puparium, so that it occupies only four-fifths of the space. The longitudinal tracheæ break their connections with the posterior spiracles, sink into the body, and disappear. Presently the anterior part of the body pulls away from the puparium and free of the larval mouth armature which is forced flat against the ventral wall of the puparium by the evagination of the head. Synchronously with this comparatively rapid process, the abdomen contracts ven-

trally so that it presses tightly against the dorsal wall of the puparium, leaving the ventral thoracic complex of wing pads and legs and the mouth parts touching the puparium, and, as a whole, clearly distinguishable from the abdomen. At this point in pupation—less than 13 minutes from its start (26°)—the head, thorax, and abdomen are all visible to dorsal view, the abdomen comparatively large. The imaginal disks of the thorax and head have been everted. Presently the legs elongate and become distinct from the wing pads and mouth parts and from one another—about 12 minutes later at 26°. The abdomen shrinks, and the pupa is fully formed, not nearly filling the puparium.

While the internal metamorphosis of *Drosophila* has never been described, it may be inferred from descriptions of the blow-fly, *Calliphora* (Kowalevsky, van Rees, Lowne, Perez,), and of the apple maggot, *Rhagoletis* (Snodgrass). The adult structures are formed from imaginal disks, distinguishable in the embryo, which develop throughout the larval stage as conspicuous internal buds. In the prepupal period of *Drosophila* this development is completed to the point of establishing the adult form.

The prepupal stage is characterized by an intense histolysis of most larval structures, such that the internal contents of the pupa just after pupation are largely fluid. This histolysis is probably nearly completed in the prepupal period. This is indicated by analogy with *Calliphora* (Perez) and by the considerably larger rate of oxygen consumption found by Bodine and Orr (1925) in first day pupæ (puparia) of *Drosophila*. Lack of agreement in temperature coefficient between rate of prepupal development and rate of oxygen consumption in *Drosophila* prepupæ (Orr, 1924–25), however, suggests that histolysis does not control developmental rate. In fact histolysis could exert such an effect only if it were to proceed at so slow a rate that the body fluids failed to supply the imaginal disks with sufficient nutrient, an improbable situation. From this evidence, therefore, and the absence of tissue differentiation upon completion of pupation, we may associate the limiting processes in prepupal development with the rate of growth and cell division in the imaginal disks, including those forming the head. In this latter characteristic, *Drosophila* differs from *Calliphora* and *Rhagoletis*, in which the head is not everted until some time after the thorax and appendages are visible. Evidence will be presented later to show that the imaginal disks probably do not control puparium formation or the rate of larval development.

The Temperature Characteristic for the Prepupal Period.

Two forms of temperature coefficient are in common use, Q_{10} and μ. The former is the ratio between the velocity of a process at temperature $t°$ and its velocity at temperature $t°-10°$. It is an empirical term without theoretical implications and has been found for many biological processes to decrease as the temperature increases. Although Q_{10} for smaller temperature intervals than 10° can be

TABLE I.
Length of Prepupal Period.

Temperature.	Experiment No.	No. of hrs. isolated.	Mean period.		Curve values.	
			Male.	Female.	Male.	Female.
°C.			hrs.	hrs.		
12.0	107c	2.0	63.00±.29	60.40±.25	63.25	60.94
12.8	106c	4.5	52.50±.09	51.31±.11	53.45	51.87
14.0	103d	2.0	43.45±.08	42.04±.12	41.68	40.37
14.9	105c	2.0	34.44±.07	33.45±.06	34.67	33.65
16.0	104a	1.0	27.22±.11	26.36±.12	27.73	26.92
16.8	107i	1.5	26.56±.07	25.83±.08	25.29	24.66
17.4	102d	1.5	23.89±.07	23.28±.07	23.88	23.23
18.3	106b	4.5	22.13±.04	21.57±.04	21.88	21.23
18.4	102a	2.0	20.81±.06	20.20±.06	21.68	21.04
19.3	103b	3.0	19.29±.05	18.83±.04	19.86	19.23
20.25	102c	3.5	18.55±.04	17.71±.04	18.11	17.54
	103a	3.0	17.63±.06	17.01±.05		
	103g	2.5	18.44±.06	18.01±.05		
	105a	1.5	18.08±.05	17.42±.06		
	105a	5.5	18.57±.03	18.09±.02		
	105b	7.5	18.26±.03	17.60±.02		
	106a	3.0	18.05±.05	17.48±.06		
	106d	1.5	17.90±.08	17.66±.07		
	107a	2.0	17.40±.04	16.96±.05		
21.24	103f	2.0	16.31±.04	15.65±.04	16.48	15.96
22.3	107b	1.5	14.58±.03	14.17±.04	14.93	14.42
23.0	103c	2.5	13.65±.03	13.16±.03	13.96	13.49
23.6	107e	1.5	13.34±.04	12.89±.03	13.21	12.76
	107e	1.0	13.28±.05	13.00±.04		
23.9	106e	4.5	12.98±.03	12.50±.05	12.82	12.39
24.1	102e	2.5	12.95±.04	12.56±.05	12.62	12.19
24.9	102b	1.5	11.79±.03	11.28±.03	11.80	11.25
26.1	103e	2.0	11.46±.03	10.83±.04	11.22	10.71
27.0	104e	2.5	10.55±.03	10.15±.04	10.76	10.30
28.2	104c	1.5	10.19±.04	9.89±.03	10.23	9.79
29.0	106f	4.5	9.78±.03	9.39±.03	9.88	9.49
30.0	104d	2.0	9.61±.05	9.08±.06	9.46	9.10
31.0	105d	3.0	9.40±.04	9.25±.03	9.07	8.73
32.0	107d	2.0	9.77±.03	9.42±.03	8.69	8.38
33.1	108a	3.5	10.20±.04	9.80±.03	8.30	8.02
20.25*	108b	7.0	19.12±.04	18.66±.05	18.11	17.54
24.8*	108c	4.5	13.11±.04	12.67±.02	11.80	11.40

* Temperature prior to puparium formation 16.7°C.

readily calculated, in practice it is seldom used for closer analysis. Recently the constant μ of Arrhenius' equation has come into greater favor both among chemists and biologists. Originally an empirical relation, it has acquired several theoretical explanations which are under active investigation by physical chemists. Aside from its probably possessing a theoretical significance, μ implies no particular
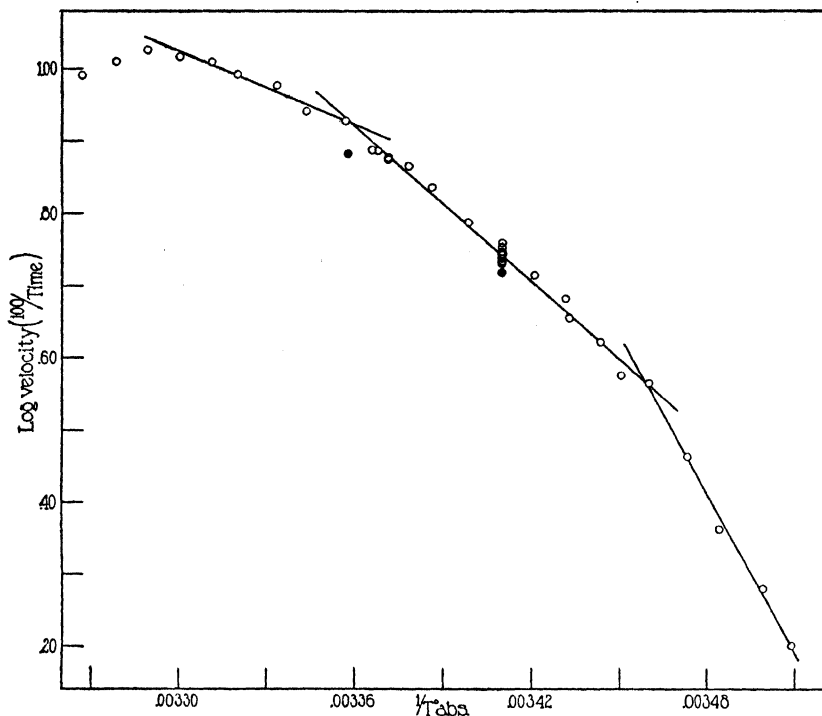
FIG. 1. The rate of prepupal development of the male, data from Table I.

temperature interval, is a much more sensitive measure, and is relatively constant over the temperature range encountered by biological processes. It is the measure here adopted.

The temperature characteristic μ is defined by the equation:

$$\log k_{T_2} - \log k_{T_1} = \frac{\mu}{4.605}\left(\frac{1}{T_1} - \frac{1}{T_2}\right)$$

in which log k_{T_2} and log k_{T_1} are the common logarithms of the velocity constants at absolute temperatures T_2 and T_1 respectively, and 4.605 the gas constant 2 multiplied by the modulus. The relation between log k and $1/T$ is rectilinear, μ giving the slope of the line, and in this form the data of Table I have been plotted, each sex separately, in Figs. 1 and 2. The experimental points very ob-
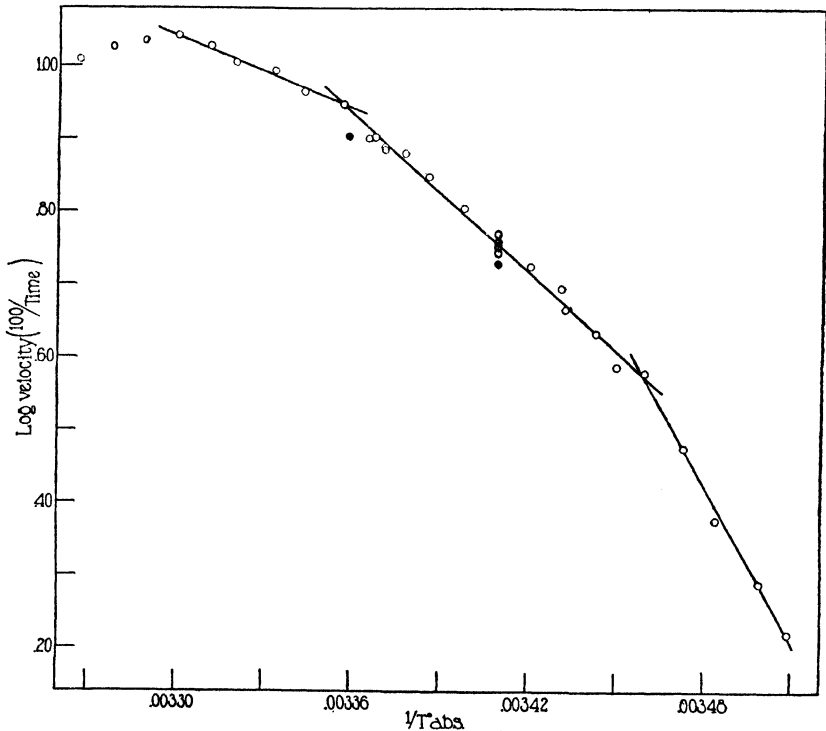
FIG. 2. The rate of prepupal development of the female, data from Table I.

viously do not fall on a single straight line, but instead seem to describe a curve. Were the relation really curvilinear, the temperature coefficient as defined by the Arrhenius equation could not be a constant, but would decrease as the temperature increased. An alternative treatment is to fit several straight lines to the points, representing different values of μ over different temperature intervals. This procedure has been used very effectively by Crozier

(1924–25), and will be followed here, with points of inflection at 16°
and 25°C. (Figs. 1 and 2). For each sex separately three straight
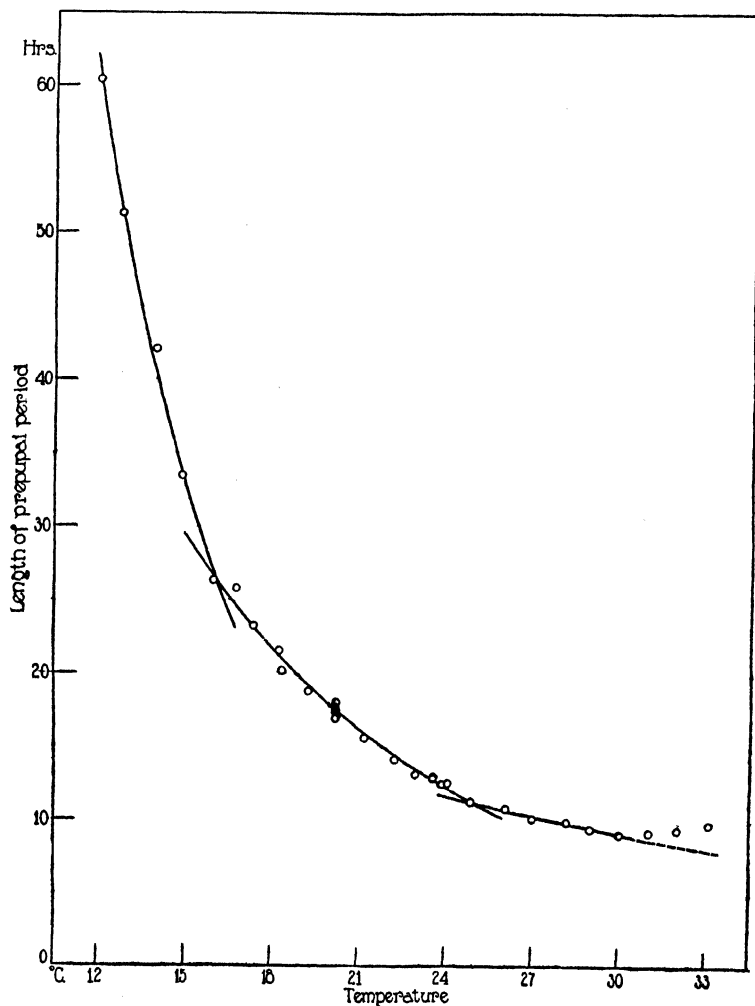
FIG. 3. Length of prepupal period in the female, curves transposed from Fig. 2.

lines have been fitted, weighting each temperature equally and using
the principle that the line connecting the partial means passes through
the mean of the whole distribution. The experimental values ap-

proximate the lines closely enough to justify this treatment, and compare favorably with most of the cases reported by Crozier.

Each value, however, is a mean of from 18 to 230 individuals (average 60) with its own probable error (Table I). Whether the probable error indicates normal variability may be tested by the coefficient of variation, $v = 100\ \sigma/\text{mean}$. If the standard deviation (σ) satisfactorily measures the inherent chance fluctuation, it should maintain a constant ratio to the mean at different temperatures. This it does, the mean coefficient of variation for the 70 cases in Table I being $3.14 \pm .06$ per cent, the two sexes the same within the limits of the probable error ($v\,\male = 3.07$ per cent, $v\,\female = 3.20$ per cent). When the difference between a given mean and the corresponding value on the fitted curve, transposed as in Fig. 3, equals or exceeds three times the probable error of the mean, it may not be attributed to variability of the material. By this test, nearly three-fourths of the means differed from the corresponding curve values by a significant amount, not including temperatures above 30°. Unless these deviations can be further analyzed, the reliability of the values for μ is considerably diminished.

Analysis of Deviations from Expected Curve.

From inspection of Table I, two variables are apparent, (1) the number of hours over which the individuals entering a given mean were isolated, and (2) the order of experimentation in relation to temperature—the successive experiments being numbered serially. If the records from only two or three successive $\frac{1}{2}$ hour isolations are added to give a basic record, the first variable is eliminated and the second variable may be tested. This has been done and the results are given in Table II.

The ratio of the experimental mean period to the curve value for each sex (Table I) gives the proportionate deviation independently of temperature. Since the two sexes show very comparable temperature relations, it seems probable that the two sexes would be affected similarly in any given experiment if the deviations can be analyzed. Correlating the proportionate deviation from male expectation or curve value with the proportionate deviation from female expectation, the correlation coefficient is $.88 \pm .02$, verifying

TABLE II.

Age of Culture and Length of Prepupal Stage.

Experiment No.	Age of culture.	Male.		Female.		Experiment No.	Age of culture.	Male.		Female.	
		Mean period.	No.	Mean period.	No.			Mean period.	No.	Mean period.	No.
	hrs.						*hrs.*				
102a	3.0	20.68	28	20.08	23	105b	33.0	18.38	25	17.63	31
	4.0	20.88	24	20.27	32		34.2	18.45	41	17.83	36
b	24.8	11.79	62	11.28	47		35.5	18.32	14	17.55	31
c	27.0	18.67	26	18.02	21		36.5	18.02	27	17.41	33
	28.0	17.92	18	17.36	26		37.5	18.30	22	17.61	27
	31.2	18.52	52	17.77	45		38.5	18.18	31	17.78	30
d	39.8	23.89	49	23.28	37		39.5	18.06	17	17.44	41
e	51.5	12.85	20	12.52	20	c	40.5	34.42	31	33.65	24
	52.8	13.02	27	12.60	20		41.5	34.46	34	33.34	44
						d	56.8	9.25	16	9.20	27
103a	1.2	17.52	20	16.98	21		58.2	9.50	26	9.28	37
	3.5	17.74	21	17.06	16						
b	4.5	19.47	15	18.93	15	106a	7.2	18.06	16	17.57	15
	5.5	19.38	12	18.98	21		8.5	18.05	21	17.38	13
	6.5	19.11	22	18.58	19	b	10.2	21.96	13	21.60	24
c	7.8	13.60	39	13.06	35		11.5	22.25	28	21.43	22
	9.0	13.71	28	13.30	23		13.2	22.05	22	21.64	28
d	10.0	43.34	38	41.92	24	c	14.8	52.84	28	51.02	22
	11.0	43.48	27	42.19	18		16.2	52.31	18	51.61	18
e	28.0	11.45	31	10.80	28		17.8	52.20	20	51.37	15
	29.0	11.48	26	10.86	31	d	31.2	17.90	19	17.66	19
f	30.0	16.25	34	15.65	23	e	32.8	13.13	19	12.83	9
	31.0	16.39	28	15.66	29		34.2	12.92	18	12.50	16
g	47.5	18.17	24	17.76	42		35.5	12.92	24	12.36	22
	48.8	18.64	31	18.21	29	f	37.2	9.62	20	9.20	15
							38.8	9.76	33	9.39	44
104a	8.0	27.22	18	26.36	29		40.2	9.96	22	9.50	28
c	32.8	10.19	61	9.89	52						
d	43.5	9.42	20	9.00	14	107a	3.0	17.42	31	16.98	32
	44.5	9.84	16	9.12	24		4.0	17.37	26	16.92	25
e	50.5	10.51	38	10.07	28	b	5.2	14.58	54	14.17	55
	51.8	10.58	38	10.23	31	c	6.5	62.31	29	59.40	20
							7.5	63.95	21	61.20	25
105a	7.2	18.08	36	17.42	38	d	8.5	9.78	27	9.34	28
	13.0	18.88	26	18.14	22		9.5	9.76	29	9.52	24
	14.5	18.42	38	17.89	41	e	22.2	13.34	46	12.89	37
	15.8	18.35	47	17.71	41		29.0	13.28	18	13.00	30
	17.0	18.56	25	18.14	39	i	33.2	26.56	25	25.83	36
	18.0	18.88	26	18.45	30	108a	1.5	10.18	22	9.86	38
							3.2	10.21	26	9.74	43

the view that the deviations exceed those expected by random sampling and indicating that their causes affect both sexes equally.

The critical increments are based upon puparia isolated over a period of 2 or 3 days each from six sets of bottles, Experiments 102 to 107 inclusive. Two variables are present: (1) differences in food conditions between the different sets of bottles, none of them being aseptic, and (2) differences correlated with age of the cultures. In no one experiment were the results strikingly different from the others, so that although the first factor possibly contributed to the irregularity in the second, it could not be measured. When the proportional deviations were plotted as a function of the time from the first isolation, the earlier lots of prepupæ were found to have developed faster than those forming puparia somewhat later, although on the second day the trend seemed in the opposite direction. Unfortunately the relation of the first isolation to the beginning of puparium formation was not recorded, but is known to have varied as much as 8 to 10 hours. Accordingly a correction factor of 6 hours for Experiments 105 and 106, and of 2 hours for 102 has been added in each instance on the assumption that the different experiments are really comparable. The results (Fig. 4) are admittedly not very satisfying, although the correlation ratio, η, corrected for too fine a grouping, is .69 $\pm$ .03. The curve of the figure is so drawn as to approximate the means calculated in determining η.

There is other evidence, however, that the successively isolated individuals from the same parents show an increasing prepupal period, primarily that of experiments on length of successive puparial stages on which the partial correlations referred to earlier are based. During 14 successive hours (at 30°) the prepupal stage lengthened consistently. In the experiments on diurnal fluctuations in emergence where puparia were isolated and timed for 50 consecutive hours, the mean length of the puparial period, plotted as a function of hour of day of isolation, was consistently longer for those isolated on the 2nd day than for those on the 1st. This result indicates that the delay in the prepupal period is not compensated for in the pupal stage. The possible causes of this effect will be considered in another connection.

Another factor causing the deviation to exceed chance fluctuation

may be inaccurate temperature control both in incubator regula-
tion and during manipulation. While the temperature during the
prepupal stage held fairly constant, that during the larval period was
not of comparable accuracy, so that all individuals compared had not
necessarily reached the same stage in development at the time of
puparium formation. Room temperature during isolation averaged
21–22°, while that of the bottles was presumably 27°. The frequent
removal of the bottles from the incubator for an appreciable time
during manipulation probably caused some variation.
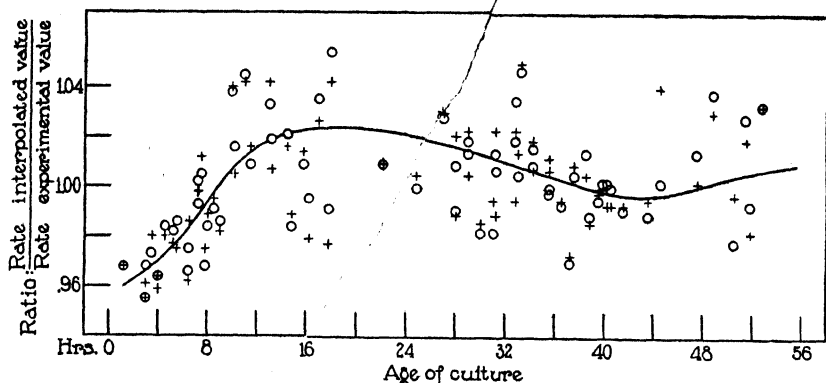
FIG. 4. Relation of proportionate deviations to age of culture, males plotted
as crosses, females as circles.

Sex Differences.

As originally determined, the temperature characteristics differ
slightly between the two sexes, −0.5 per cent (12–16°), 1.4 per cent
(16–25°), and −4.6 per cent (25–30°) on a male basis. If this can
be attributed to experimental error, the relative rates of develop-
ment of the sexes should be constant within the temperature range
fitted by a single straight line. If they show a shift with temperature
which exceeds the normal variability, the difference in the critical
increments is probably significant.

The ratio between the rate of female development and rate of
male development, calculated from Table II, has been plotted in
Fig. 5 as a function of temperature. Although the diagram shows
great variability at all temperatures, a slight increase is apparent in

the developmental rate of the female relative to the male as the temperature rises. Whether this is significant within a temperature range fitted by a single value of μ or only between such ranges, if even then, is the determining factor. The weighted mean values of the ratio for the lower and upper parts of each temperature interval are given in Table III. The differences within the lower (12–16°) and upper (25–30°) ranges are as expected from the determinations of μ, but they fall within the limits of the probable error of the ratio for each total range (p.e. = .002 +). The corresponding values of μ,
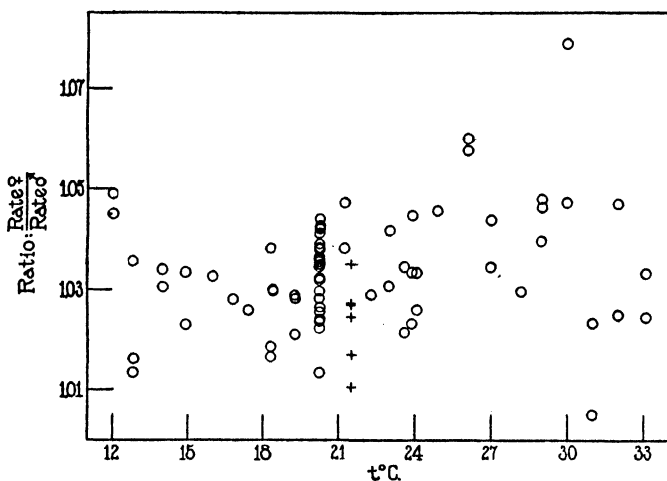
FIG. 5. Developmental ratio of the sexes.

therefore, are not significantly different. Within the longer middle range the result is less certain: the differences exceed their probable errors but not significantly. The larger value of the ratio in the upper as compared with the lower temperature zone (difference = .015 ± .004), however, may be connected by a continuously increasing velocity of female development in the intermediate interval. The data are inconclusive, but for conversion to a male basis in the next section, the shift will be assumed.

The difference between males and females in developmental rate, long familiar to *Drosophila* geneticists, is here shown to appear as early as the prepupal stage. The evidence is clear-cut, all but 4 out

of 80 cases falling within a range in developmental ratio of 1.01 to 1.05, a variation of but 4 per cent.

Corrected Temperature-Development Curve.

The deviations of the experimental points from the curves of Figs. 1 and 2 have been correlated with age of the cultures at the time of puparium formation. If this factor were eliminated, would the points approximate more nearly to three straight lines when plotted as in Figs. 1 and 2? It is not unreasonable to apply such a correction to the present data for two reasons. (1) As analyzed in Table II, 37 per cent were means of prepupæ raised at 20°. This proportion

TABLE III.

Mean Developmental Ratio of the Sexes for Different Temperature Intervals.

Temperature range.	Mean ratio.	Temperature range.	Mean ratio.	Temperature range.	Mean ratio.
°C.		°C.		°C.	
12–13	1.033	16–19	1.027	25–27	1.048
14–16	1.031	20	1.033	28–30	1.046
12–16	1.032	21–25	1.035	25–30	1.047
		16–25	1.032	30–33	1.036

would probably suffice to scatter the points even more than they are if the true correction were very different from one based on Fig. 4. Means deviating widely from the curve of Fig. 4, when corrected from the curve, might very possibly give a poorer fit to the Arrhenius equation at critical points than do the crude data. (2) Granting that the correction is rough and uncertain, it is much superior to no correction. As pointed out by statisticians, crude data which include a known error are not unweighted, but are weighted inaccurately, more inaccurately than those treated with the roughest sort of correction.

Before applying the correction for age of culture, however, all the female records of Table II have been converted to a male basis by means of the mean developmental ratio of the sexes, thus doubling the number of cases. Following this the partial means of Table II have been corrected for the age of culture error from Fig. 4, and the

results plotted in Fig. 6, males as crosses, females as circles. In order to calculate the temperature characteristics, a weighted final mean for each temperature was determined (plotted in Fig. 7). These means should give the most reliable values of μ which can be secured from the data. In lieu of the reciprocal of the probable error, each value was weighted by the square root of the number of cases on

FIG. 6. Final curve for rate of prepupal development, showing partial means.

which it is based, and the μ calculated. The method, the same as that used before, may be described in more detail. Each temperature range is divided into an upper and lower part and the weighted mean values for logarithm of the velocity and reciprocal of the absolute temperature determined for each part. From these two values, μ can be calculated arithmetically and the line placed on the diagram. Since all the points did not fall on the line, the value for

μ was determined twice, in one case with the mid-value of a given interval in the upper part mean, in the other case with it in the lower part mean, and the two averaged. The values for 16° and 25°, the critical points, were included in calculating the μ for the temperature ranges both above and below them.
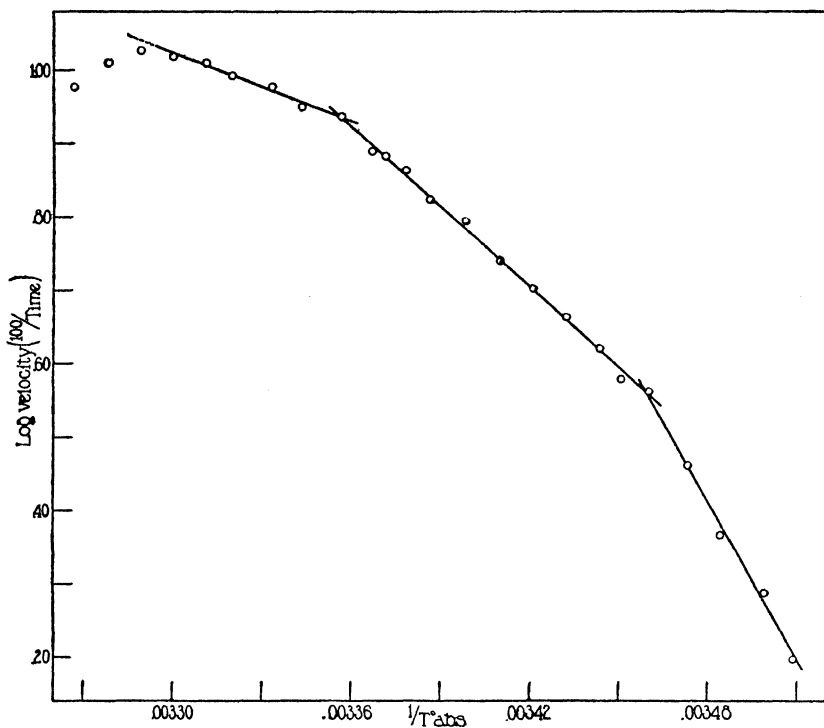
FIG. 7. As Fig. 6, but with final weighted means from which the curve was calculated.

The final temperature characteristics are given in Table IV, and apply to either sex, except in the middle temperature region where the value of μ for the female is 0.7 per cent greater than for the male (the one given). Probable errors would be of doubtful value since the error in the age of culture correction is unknown. Accordingly Table IV does not tell us how significant are the differences between the critical increments. The only index to the excellence of fit is by

examination of the figures. The experimental values up to 30° seem to me sufficiently well described by the Arrhenius equation to warrant its use. Any interpretation of the processes controlling the developmental rate of the *Drosophila* prepupa must be consistent with this relation.

Does a Given Critical Increment Apply throughout the Prepupal Period?

The Arrhenius equation has been applied to the development of the prepupa under constant temperature conditions, and suggests that a single rate-limiting master process, characterized by its critical thermal increment, may extend throughout the period at any given temperature. If this is true, the μ for a part of the period should

TABLE IV.

Temperature Characteristics for the Prepupal Period.

Temperature range.	Original data.		Corrected data.
	Male.	Female.	
°C.		.	
12–16	33,850	33,680	33,210
16–25	16,440	16,670	16,850
25–30	7,880	7,520	7,100

agree quantitatively with that for the whole. This was tested in two preliminary experiments similar to those described in an earlier paper.

In the first experiment with 20° as the standard temperature, the prepupæ consisted of two groups. Those of one group were exposed for 10 hours at 14° in three sets at the (1) beginning, (2) middle, and (3) end of the prepupal stage. For the rest of the period they were kept at the standard temperature and the time of pupation noted in each case. The other group was similarly treated to 3 hours exposure at 26°. In each case the temperature difference included a break in the curve, a fact not appreciated at the time. The controls developed at constant temperatures of 14°, 20°, and 26°.

In computing the μ for the different parts of the period, the length of exposure to 14° or 26° was compared with the length of the equiva-

lent stage in those kept constantly at 20°. This assumes that the stages passed at 20° in the experimental animals lasted as long as equivalent stages in the controls. Then

$$M_c - (M_x - D_x) = D_c$$

when M_c = mean prepupal period of control (at 20°), M_x = mean period of experimental lot (at 14° or 26°), D_x = duration of exposure to changed temperature, and D_c = duration of equivalent stages at control temperature (20°). Substituting the reciprocals of D_x and

TABLE V.

First Experiment on Temperature Characteristics for Parts of Prepupal Stage, Standard Temperature 20°.

Treatment.		Mean period.		Thermal increment.	
		Male.	Female.	Male.	Female.
hrs.	*°C.*				
1–10	14	24.347±.052	24.056±.051	26,800±520	29,000±560
6–15	14	24.227±.068	23.815±.053	25,940±590	27,160±550
11–21.8	14	25.666±.091	24.766±.118	28,340±680	26,330±780
11–20.2	14	24.921±.086	24.159±.088	29,960±790	28,560±800
Entire	14	45.156±.150	44.000±.108	25,690±120	25,650±100
period	20	18.190±.049	17.609±.050		
at	26	12.214±.068	11.849±.034	11,650±190	11,590±120
1–3	26	16.394±.066	15.672±.049	13,700±500	14,550±410
6–8	26	16.100±.051	15.794±.042	15,440±410	13,820±390
6–8.5	26	15.929±.086	15.393±.080	14,560±510	14,320±480
11–13	26	16.603±.059	16.122±.058	12,400±490	11,750±500

D_c in the Arrhenius equation, the temperature characteristic can be readily determined. Since both M_x and M_c are averages with probable errors, the probable error of D_c is readily secured. Two values of μ were determined in each case, one from D_c + p.e., the other from D_c − p. e., and the mean of these taken as the true value.

The results of the experiment (Table V) are quite erratic, and most of the differences of questionable significance. Much of this can be attributed to experimental error, the numbers being small and the marked variation correlated with age of the culture uncorrected. The

sex differences fluctuate so irregularly as to be meaningless. When compared with the standard curve of Fig. 7, the developmental rates of the controls were all markedly low, and unequally so, giving smaller values of μ for the upper temperature range and larger values for the lower range than standard. Yet for both groups, the temperature characteristic for the whole as determined from the controls was less than that for any of the parts.

TABLE VI.

Second Experiment on Temperature Characteristics for Parts of Prepupal Stage, (No. 107e to i), Standard Temperature 23.6°.

At 16.8°	Age of culture.	Mean period.		Average period. Male basis.	No.	Age correction.	Final mean, M_x.	D_c for D_x = 6 hrs.	μ
		Male.	Female.						
hrs.	*hrs.*								
0–6	25.5	16.61	16.21	16.615±.010	88	1.012	16.429±.031	2.694	20,220
	32.0	16.95	16.50	16.932±.019	41	1.028		±.042	±390
5.5–11.5	27.5	16.63	16.06	16.542±.029	101	1.014	16.270±.058	2.853	18,770
	30.0	16.37	16.09	16.428±.025	35	1.019		±.064	±570
9–15	23.8	16.10	15.93	16.211±.095	71	1.012	16.009±.080	3.114	16,570
	31.0	16.37	15.94	16.349±.018	39	1.023		±.085	±690
23.6°*	22.2	13.34	12.89	13.283±.023	83	1.011	13.123±.028		
	29.0	13.28	13.00	13.308±.015	48	1.016			16,870
16.8°*	33.2	26.56	25.83	26.514±.027	61	1.036	25.593±.027		±60

* Controls, temperature constant throughout.

The second experiment proved much more satisfactory. The standard temperature was 23.6° and treatment lasted 6 hours at 16.8°. Thus the experiment fell within the range characterized by $\mu =$ 16,850. The pronounced age of culture error could be corrected because of the continuity of the data: the developmental ratio of the sexes was sufficiently uniform that a weighted mean ratio (1.025) permitted conversion of all records to a male basis: and fair sized numbers reduced the probable error to within bounds. The calculations followed the same principles as in the preceding case; the results are given in Table VI.

The differences in μ are probably significant. If verified, they would indicate that several successive reactions limit the rate of development, and that μ for the whole averages a time series. The thermal increment is greatest, however, during the first part of development and least at the end. Further, μ for the control is not intermediate in value between the lowest and the highest, but within the limits of the probable error is identical with that for the end of prepupal development. This does not agree with the view that the critical increment for the whole averages a time series. Nevertheless, the differences in duration of the prepupal stage due to time of incidence of the lower temperature are probably real.

The discrepancy may arise from the basic assumption upon which the μ are calculated; *i.e.*, that the effect of the lower temperature is confined to the stages exposed to it. Instead the effect of the lower temperature may persist after the prepupæ are returned to 23.6°. The stages actually exposed to the low temperature would then be delayed only as much as the value of μ determined for the whole period would demand. The mechanism for producing such an effect is not evident from the data.

We may conclude from these experiments that while fluctuations in end-point as functions of time of exposure to a changed temperature are real, they indicate only that the temperature characteristic for the whole period cannot be the simple average of the μ for a series of successive reactions differing in their critical increments. This type of experiment, however, seems more likely to provide an analysis of dynamic interrelations in development than constant temperature studies.[1]

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[1] In conversation, Dr. Crozier has suggested that if the velocity constant of the autokinetic growth reaction were the sum of two factors, one the constant characteristic of the main reaction, the other that expressing the catalytic effect of a resultant, the velocity curve for the entire process would have different forms at different temperatures (unless the temperature coefficients of the two components were identical). This would explain the shift in end-point described here when the relative period of incidence of a lowered temperature is changed. Although the interpretation is complicated, data secured under constant temperature conditions might still apparently agree with the Arrhenius equation within the limits of the experimental error.

Effect of Temperature Prior to Puparium Formation.

Inaccurate temperature control prior to puparium formation has been named as one source of experimental error. How important is this factor? A set of culture bottles, raised at the standard temperature of approximately 27°, were put at 16.7° when they started to produce puparia. After 22 hours at the lower temperature prepupæ were isolated at $\frac{1}{2}$ hour intervals for 11 hours. The first of these passed the prepupal stage at 20.25°, the last at 24.8°: the results are given at the end of Table I and plotted in Figs. 1 and 2 as solid black circles. While the length of the prepupal period is shown to be conditioned by the temperature prior to its onset, the results are not sufficiently accurate to determine the temperature characteristic of this delay. The greater difference from standard of the 24.8° group is probably caused by their longer exposure to the low temperature before puparium formation.

This experiment puts one on guard in dealing with markers of development. Whether one would find the same increments and critical temperatures for the prepupal period following some temperature other than 27° cannot be predicted. Nor are such data comparable with those in which the larval period is passed at the same temperature as the pupal period, as in the studies by Loeb and Northrop.

The delay in the prepupa following the lower temperature at the end of the larval stage is not unexpected. If puparium formation is conditioned by larval growth independently of imaginal disk development, a smaller temperature coefficient for larval growth than for imaginal disk development would give the observed results. The puparium would form before the disks had reached as advanced a stage as under a larval temperature 10° higher. Published data on *Drosophila* larvæ are not detailed enough to decide the point.

This supposed independence of larval and imaginal processes is a very suggestive viewpoint. It is consistent with Baumberger's results (1919) on the effect of concentration of yeast in larval food upon length of the larval and pupal periods. Although he concludes that the two stages are independent, his Fig. 8 seems to show a negative correlation between them. This would be expected if the de-

ficient food supply at low yeast concentrations were not strictly apportioned between larval growth and the much smaller requirements for imaginal disk development.

The lengthening of the prepupal period as the culture from which the puparia are isolated grows older seems inconsistent. Baumberger found that larger pupæ were formed from better fed larvæ, which in turn developed faster. The first puparia to form in a culture are largest, and on this ground should be the best nourished, have the shortest larval period, and consequently the longest prepupal period if the age change can be attributed to nutrition. The food conditions in the cultures change from day to day and would seem to furnish the necessary mechanism. J. C. Li[2] has shown, however, that larvæ from the first eggs to be laid had the longest larval period, while those from later eggs required a constantly decreasing time to complete their growth over a 6 day oviposition period. The length of the puparial stage of the same individuals, however, increased to a corresponding degree, agreeing with the condition in the prepupæ. Li has further demonstrated that this change was not correlated with the condition of the culture medium of the larva, which was constant for all individuals, but must be traced back to conditions under which the successive eggs are developed in the mother. The age of culture change is consistent, therefore, with the explanation adopted here. If it is sufficient alone, the age change in the larva must be completely accounted for in the prepupal stage, a demonstration that has yet to be made.

If the effect of a lowered temperature at the start of the prepupal period holds over after return to a higher temperature, there is no reason why such a change just before the prepupal period starts should not have the same effect. The effect of this hold-over is quantitatively not sufficient, however, to account completely for the results obtained.

<div align="center">DISCUSSION.</div>

The interpretation of temperature characteristics similar to those presented here is not self-evident. Crozier has frequently used the catenary system of limiting reactions $A \xrightarrow{k_1} B \xrightarrow{k_2} C$ to explain his results.

[2] Unpublished data.

Each reaction in such a series is characterized by a value of μ determined experimentally. At the critical temperature, where a break occurs in the plotted data, the velocity coefficients k_1 and k_2 are equal. At higher temperatures the reaction with the smaller thermal increment is the slower, and as the limiting process its temperature characteristic is that of the process as a whole. As the temperature falls below the critical point, however, its velocity does not diminish as rapidly as that of the reaction characterized by a larger value of μ. This latter is then the limiting reaction, and its characteristic represents the process as a whole. He further assumes "that the critical increment refers to . . . . the formation of active molecules or ions of a catalyst." The velocity of the biological process on this basis is that of the limiting reaction, which proceeds in the direction of the process.

A recent paper by Janisch (1925), however, interprets the decreasing acceleration at higher temperatures on quite a different basis. He postulates a reverse reaction, opposite to the anabolic process that dominates insect development at lower temperatures. His experimental data are inadequate for a critical test, however, and his analysis is not based on physicochemical principles. In 1914, Pütter proposed the same idea that the velocity of a biological process at the higher temperatures is a resultant of two opposed reactions, one promoting the function, the other retarding it. Pütter's analysis has been corrected and extended by Hecht (1918–19), who used it very successfully in explaining the effect of temperature upon the photosensory latent period of *Mya*. However, the hypothesis as corrected by Hecht has not been applied to insect development which both Pütter and Janisch explain on this basis.

For temperatures from 25–30°, I have assumed that the rate of prepupal development is adequately described by the fitted line, and, using Hecht's analysis, have determined the corresponding velocity constants for the reverse process (k_2). Let us assume in the reactions $A \xrightarrow{k_1} B$, $B \xrightarrow{k_2} C$ that a certain intracellular concentration of B must be reached before any given cell in the imaginal disks will divide, and that its formation is characterized by $\mu = 16{,}850$. Above 25° we will postulate a destructive process which breaks B down into an

innocuous substance C. The formation of B in a concentration necessary for initiating cell division is thus delayed. The resultant of the two might possibly give an apparent increment of 7,100, which has so far been treated as typifying an irreversible anabolic process rather than an equilibrium. If the thermolabile component, B→C, were a valid postulate, however, its temperature characteristic would be a constant. As shown in Table VII, this is not the case. If the experimental points give a rectilinear relation on the ordinates used here, the rate of the process cannot be controlled by an equilibrium between such opposing reactions. This fact has already been pointed out by Crozier, but does not seem to be appreciated sufficiently. Above 30°, however, where the relation is not linear with these coordinates, this explanation may very well apply.

TABLE VII.

Velocity Constants, k_2, and Thermal Increments, μ, for Hypothetical Thermolabile Reaction.

Temperature.	k_2	μ
27	.00130	
28	.00197	76,300
29	.00271	57,900
30	.00348	45,400

The thermal increments characterize three distinct anabolic processes which probably limit the rate of cell growth and, possibly, cell division in the imaginal disks of the head and thorax. Certainly one cannot invoke a mechanism, such as Robertson's autocatalytic theory of growth, which postulates a reversible limiting reaction that progresses to an equilibrium. The limiting reactions need not be specific determiners of pupation which terminates the prepupal period. More probably pupation is initiated by pressure or other mechanical forces due to crowding of the cells in the invaginated imaginal disks. Since these are already well developed by the end of the larval period, the prepupa represents a relatively uniform stage of development. Even so, the short exposures to a lowered temperature already described showed that the successive parts were not entirely equivalent in their temperature relations.

A catenary chain of consecutive simultaneous reactions has been used to explain the three values of μ: A→B→C→D. Whether such a system could give the observed results is questionable. As suggested by C. R. Plunkett,[3] at temperatures such that B→C (or C→D) is the slowest reaction of the series, B would tend to accumulate faster than it would transform to C, and thereby accelerate B→C by the law of mass action. The temperature characteristic of the entire process in this case could not be that of the component B→C, but would be governed primarily by A→B. By postulating that each reaction to the left of the slowest member is reversible, this difficulty may be partly obviated. At temperatures where A→B is the slowest component, B would be removed as rapidly as formed, so that B→A would be negligible. At other temperatures where B→C is the slowest component, A⇌B would maintain B in constant concentration dependent upon the temperature, so that B→C could not be continuously accelerated by mass action. The temperature coefficient, however, would average the μ for B→C and the effect of temperature upon the equilibrium A⇌B. It could not have a relatively simple meaning, and it is doubtful if the Arrhenius equation would fit it.

As an alternative, the temperature characteristic may be attributed to a series of relatively independent reactions.[4] Here constant
$$A→B\searrow$$
minimal concentrations of B, D, and F C→D——⟶K are required to
$$E→F\nearrow$$
complete the cycle between equivalent stages in successive cell divisions. Then the three reactions might exhibit so loose an interdependence that the rate of the whole would be that of the slowest component and would be characterized by its value of μ. This proposal seems to avoid the dynamic difficulties inherent in the others and suggests a morphological basis. During interkinesis, to which the limiting processes have been assigned, a large number of more or less independent processes must reach a given stage before the mitotic
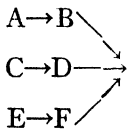
[3] Personal communication.
[4] This possibility, I find, has already been suggested by Crozier (1924–25, p. 213) in somewhat different form.

mechanism is released. Thus the formed bodies of the cytologist, particularly the chromosome elements, are double in the early prophases. Accordingly the limiting reactions may be attributed to those three (in this case) which require the most time to grow duplicates of themselves.

The values of μ obtained here may be compared with those of other processes (Table VIII). The prepupal stage does not agree either in values of μ or in critical temperatures with determinations for the

TABLE VIII.

Temperature Characteristics of Some Related Phenomena.

Object.	Observer.	Temperature range.	μ
		°C.	
Drosophila prepupa (rate of development).	Bliss.	12–16	33,210
		16–25	16,850
		25–30	7,100
Drosophila pupa* (rate of development).	Loeb and Northrop.	15–20	27,000
		20–30	10,000
Drosophila egg* + larva (development).	Loeb and Northrop.	10–20	27,000
		20–30	10,000
Drosophila prepupa and pupa (O_2 consumption).	Orr.	1–15	16,800
		15–30	11,500
Tenebrio pupa* (rate of development).	Krogh.	14–22	27,000
		22–30	10,000
Arbacia egg* (rate first cleavage).	Loeb and Wasteneys.	7–11	41,000
	Loeb and Chamberlain.	11–19	21,000
		20–27	12,400

* Determinations of μ quoted from Crozier.

entire puparial period of which it is a part. The increment for the puparial period therefore averages a time series, but the number of determinations is so small that the temperature characteristics based upon them are of little significance. The same is true for the egg + larval stage. When a process is known to consist of several, quite different, successive phases, apparent agreement with the Arrhenius formula is no assurance that the μ derived applies to a single extended limiting reaction. The pupal period of *Tenebrio* (Krogh, 1914), which gives a beautiful fit below 22° to the Arrhenius equation, is probably equally heterogeneous. At least it includes the same high

initial CO_2 production (Krogh's figures) demonstrated by Bodine and Orr in *Drosophila* and here identified with the prepupal period.

A more curious circumstance is the quantitative agreement between the critical increments for O_2 consumption of the *Drosophila* pupa (Orr, 1924–25) and rate of prepupal development. However, the former holds *below* 15°, and the latter *above* 16°; at equivalent temperatures the two are distinctly different. That the same reaction limits the one process of respiration below 15° and the other of speed of development above 15° is questionable.

Roughly, the increments for the prepupa form a geometrical series, a puzzling relationship that is not uncommon; for example, the values for the first cleavage of *Arbacia* (Loeb and Wasteneys, 1911; Loeb and Chamberlain, 1915).

SUMMARY.

1. Diurnal fluctuations in emergence of the adults and negative correlation between the length of successive stages in the puparium made it desirable to restrict study of relation of temperature to development to the prepupal stage.

2. On morphological grounds, the formation of the puparium, which starts the prepupal period, seemed to be determined by the stage of larval development; pupation, which terminates the prepupal stage, by imaginal disk development.

3. The rate of prepupal development may be represented by the Arrhenius equation relating velocity of an irreversible chemical reaction with temperature. The data gave three values for the critical increment over different temperature intervals, corresponding to three straight lines of different slope. When deviations of the points from these lines were compared with their probable errors, however, in nearly three-fourths of the cases the difference was significant.

4. Analysis of these deviations showed them to be due primarily to changes in the extent of imaginal disk development at the time of puparium formation. These, in turn, were correlated with age of the culture.

5. The two sexes differed in developmental velocity, such that the rate of female development was about 1.03 times as great as rate of male development. For the upper temperatures this ratio was

greater than for the lowest of the three temperature ranges, the intermediate zone possibly varying between the two.

6. A final curve relating prepupal development to temperature has been calculated after (1) converting all female records to a made basis, (2) applying a correction for age of culture error, and (3) weighting each point by the square root of the number of cases upon which it was based. This yielded the following values for the temperature characteristic μ; namely, 33,210 from 12–16°, 16,850 from 16–25°, and 7,100 from 25–30°. Above 30° the data could not be fitted by the Arrhenius equation.

7. By treating prepupæ in different developmental stages to brief exposures at a lower temperature, pupation was more delayed by treatment at the beginning than at the end of the prepupal stage. From these data, μ for parts of the stage were calculated on the assumption that the effect of temperature did not persist after return to the standard temperature. Since the μ thus secured were greatest for the beginning and least for the end, and none were less than that for the whole, the interrelations of the successive stages are probably more complex than they were assumed to be in making the calculations.

8. Lowering the temperature prior to puparium formation lengthened the prepupal stage. Puparium formation, therefore, was not conditioned by imaginal disk development, but by larval processes possessing a lower temperature coefficient than did the imaginal disks.

9. Possible physicochemical mechanisms for producing these results are discussed, and the observed temperature characteristics were finally attributed to three relatively independent anabolic processes which limit the rate of cell growth in the imaginal disks.

10. Both the thermal increments and the critical temperatures for the prepupal stage differed markedly from those reported by Loeb and Northrop for the entire development within the puparium. Since the prepupa forms part of the latter period, temperature characteristics for extended developmental phases known to be heterogeneous are of doubtful significance.

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THE RESISTANCE OF LIVING ORGANISMS TO DIGESTION BY PEPSIN OR TRYPSIN.

By JOHN H. NORTHROP.

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The fact that living organisms are not digested by pepsin or trypsin, whereas dead organisms are rapidly digested, has attracted the attention of biologists and physiologists for many years. The early work was centered chiefly about the defense of the digestive tract, but it was soon recognized that all living matter was resistant to these enzymes. The problem is evidently closely connected with the resistance of the living cell to autolysis by the enzymes already in the cell. It differs from the latter in that by using concentrated solutions of the proteolytic enzymes the digestion of dead cells may be made very rapid in comparison to autolysis. There is a much more striking difference, therefore, in the behavior of dead and living cells when exposed to trypsin or pepsin than in the autolysis of dead and living cells. Only the resistance to pepsin and trypsin is considered in this paper.

Fermi[1] was able to show in a series of careful and ingenious researches that the theory of a protective mucus or epithelium of Claude Bernard, Pavy's theory of the alkalinity of the tissues, Gaglio's theory of the absorption of the enzymes, and the antienzyme theory of Wieland were all insufficient to fully account for the results. There is no doubt that the alkalinity of the blood and the presence of antienzymes have an effect on the process, but, as Fermi points out, they are insufficient to account for the complete absence of digestion of living cells. Fermi concluded that the configuration of the protein molecule in the living cell was different from that after the death of the cell, and that the "living molecule" could not be attacked by the enzyme. This theory is not very different from Hunter's "living

[1] Fermi, C., *Centr. Bakt., 1. Abt., Orig.*, 1910, lvi, 55.

principle" except that the attribute of "living" is applied specifically to the protein molecule. Fermi considered that the assumption of a mechanism which prevented the enzyme from entering the living cell was untenable, since there are innumerable types of cell membranes, and it is not reasonable to suppose that they are all impermeable to the enzyme while the cell is living and permeable after the death of the cell. It is evident, however, that if there were such a mechanism it would account for the failure of the enzymes to destroy the cell. The work of Osterhout[2] and others has shown that the permeability of the cell is one of the most characteristic attributes of the living cell and that this permeability changes in a remarkable way when the cell is dead or injured, so that it is not at all unlikely that the permeability to enzymes is also greatly different in the living and the dead cell. It is known also that pepsin and trypsin, at least, are secreted in an inactive form. The present experiments were undertaken, therefore, to determine whether or not pepsin and trypsin actually did enter the living as well as the dead cell. It was found in every case that as long as the cell was alive, no detectable quantity of enzyme was taken up; whereas when the cell died the enzyme was rapidly removed from solution and concentrated in the cell.

Effect of Trypsin on Living and Dead Organisms.

The results of several experiments in which living and dead organisms were exposed to the action of a powerful dialyzed trypsin solution are shown in Table I. They confirm the results of Fermi and other workers, in showing that the living cell is not attacked whereas organisms which have been killed by heat or mechanical injury are rapidly digested. The results were more striking than the figures show, since in some cases the dead organism was almost completely disintegrated although the formol titration increase was small. It may be added that the animals lived just as long in the active enzyme solution as in the inactivated enzyme. The experiment also shows that the slow digestion due to autolysis is negligible compared to that due to the trypsin.

[2] Osterhout, W. J. V., Injury, recovery, and death, in relation to conductivity and permeability, Monographs on experimental biology, Philadelphia and London, 1922.

The digestion of the organisms killed by heat could be readily accounted for by assuming a change in the chemical nature of the proteins or by the destruction of the antienzyme.   These objections, however, do not apply to the digestion of the organism when killed simply by mechanical injury.   It is difficult to imagine that this would cause a change in the chemical nature of the protein, unless Fermi's "living molecule" is assumed.   The antienzyme is present and in the case of earthworms its action is marked, yet, owing to the large excess of enzyme, the tissue digests.

TABLE I.

Effect of Trypsin on Living and Dead Organisms.

Organisms added to concentrated dialyzed trypsin and left at 20°C. for 24 hours. Increase in formol titration and visible digestion determined.

	Appearance and increase of formol titration, 0.1 N NaOH per cc. solution after 24 hours.					
	Living organisms.		Killed by cutting.		Killed by boiling 10 min.	
	Active trypsin.	Inactive trypsin.	Active trypsin.	Inactive trypsin.	Active trypsin.	Inactive trypsin.
Earthworm*.....	0.8, alive.	0.9, alive.	3.50, nearly all digested.	1.0	3.50	—
Euglena.........	— , "				Cells dissolved.	
Yeast...........	— , "				0.75, cells dissolved.	
Fundulus........	0.1, "	0.1, "	0.3, partially digested.	—	0.8, partially digested.	0.10
Meal worm†....	0.1, "	—, "	2.0	0.8	0.4	—
Goldfish‡.......	—, "	—, "	0.20	—	0.35	—

* *Lumbricus terrestris.*   † *Tenebrio molitor.*   ‡ *Carassius auratus.*

Permeability of Dead and Living Tissue to Pepsin and Trypsin.

In order to see whether the enzyme can penetrate into the organisms the experiments were repeated with the modification that the organisms were placed in a small volume of the enzyme solution.   This is necessary since otherwise the change in concentration of the supernatant enzyme solution, on removal of a small quantity of trypsin, would be too small to measure.   The concentration of the enzyme in the supernatant solution was then determined at intervals by the vis-

cosity method described by Hussey and the writer.[3]   Since the volume of the enzyme solution is known, the total amount of enzyme removed can be calculated.   In every case the dead organism removed large quantities of the enzyme from solution, while the living organism removed little or none (Table II).   Pepsin and trypsin therefore cannot enter the living tissue.   It might be objected that even though it can not enter, the enzyme should attack the surface of the cell.   There is considerable reason to suppose that the surface of the cell is not protein in nature, but even though hydrolysis did take place at the surface,

TABLE II.

Removal of Trypsin from Solution by Living and Dead Organisms.

Material washed with dilute trypsin solution and placed in an equal volume of dilute trypsin at 0°C. for 24 hours.   Supernatant solution tested for trypsin.

	Total units trypsin removed from solution.				
	Living.	Killed by cutting.	Killed by boiling.	Killed by HCl.	Killed by 50 per cent alcohol.
B. coli..........	0		4.0		
Earthworm......	1.0	10.0	14.0		
Euglena........	0		6.0		
Meal worm.*....	4.0	6.0	7.0		
Goldfish........	0		4.0		
	Pepsin.				
Earthworm......	0	6.0	8.0	10.0	4.0

* Worms injured, some motion but do not recover on removal from solution.

it would be so slow compared to the rate when the enzyme was distributed throughout the cell that it could not be detected experimentally. This may be seen from the following approximate calculation.   Suppose a block of protein, $1\mu = 1000$ $\mu\mu$ cube, consists of protein molecules which are $10\mu\mu$ cube.   The block will contain 10^9 $\mu\mu^3$ or $\dfrac{10^9}{10^3} = 10^6$ molecules.   The surface layer of the block will contain only $\dfrac{6 \times 10^6}{6 \times 10^2} = 10^4$ molecules.   The rate of digestion of the block from the

[3] Northrop, J. H., and Hussey, R. G., *J. Gen. Physiol.*, 1922–23, v, 353.

surface only will be, therefore, $\dfrac{1}{100}$ of the rate if the whole block were attacked. The difference will be still greater owing to the actual concentration of enzyme inside the particle, and becomes rapidly greater as the cell becomes larger.

It may be shown by the following experiment that the enzyme is really taken up in an active form and is not merely inactivated or destroyed. Pieces of earthworms which have been killed by mechanical injury or heat are placed in a strong trypsin solution for 1 hour. The trypsin is rapidly removed from solution but in this time no noticeable digestion occurs. The tissue is then removed from the enzyme solution, washed with water, and placed in a small volume of water. After 24 hours at 20°C. the tissue is practically completely digested, while control pieces treated in the same way with inactivated trypsin are unchanged.

If the lack of hydrolysis of living cells were really due to the protective action of the membrane, it should be possible to show that digestion occurs if the enzyme is actually injected into the cell. This experiment has been performed with *Amœba*, using Chambers' microinjection technique.[4] The following are typical experiments.

1. Several *Amœba* placed in active and in inactivated 5 per cent dialyzed trypsin remain normal more than 6 hours.

2. *Amœba in active trypsin.* Entire cell sucked into capillary pipette and then blown out. Mass of granules embedded in jelly-like protoplasmic mass. This mass is rapidly digested and after 4 to 5 minutes only the granules are left.

A volume of active trypsin equivalent to about 1/3 to 1/4 the volume of the cell was injected. Rapid streaming commences, the injected solution collects into a spherical blister containing granules. The cell contracts in an "attempt" to pinch off this blister. The streaming stops and, although the blister may be pinched off, the cell becomes motionless and disintegrates in the course of a few hours. The membrane of the blister is not attacked and the spherical shape may be retained for some time.

[4] Chambers, R., *Proc. Soc. Exp. Biol. and Med.*, 1920–21, xviii, 66; *J. Gen. Physiol.*, 1922–23, v, 189.

Injection of the same trypsin solution inactivated by heat results merely in the rapid pinching off of the blister, as described by Chambers in the case of oil drops.

Further confirmation of the idea that the resistance of living organisms to external enzymes is due to the cell membrane may be found in the fact that the injection of lipase into living organisms causes rapid hydrolysis of the extracellular fat.[5]

Mechanism of the Concentration of the Enzyme in Dead Tissue.

It has long been known that coagulated proteins remove pepsin or trypsin from the surrounding solution. It was found by the writer that the relative concentration of the enzyme inside and outside of such protein particles was the same as that of other ions whose distribution was determined by the Donnan equilibrium. It seems necessary to assume, therefore, that the enzymes are ions and that their distribution is determined in the same way as that of other ions. There seems every reason to suppose that a piece of dead tissue acts in the same way as a particle of denatured protein and that the mechanism regulating the distribution is here also the Donnan effect.

SUMMARY.

1. Pepsin and trypsin are quickly removed from solution by dead organisms. They are not able to penetrate into living organisms.

2. Trypsin injected into a living *Amœba* results in the death and disintegration of the cell.

[5] *Cf.* Wells, H. G., Chemical pathology, Philadelphia and London, 3rd edition, 1918, 385.

TEMPERATURE AND LOCOMOTION IN PLANARIA.

BY WILLIAM H. COLE.

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I.

In order to secure further information on the temperature characteristics of vital processes, as determined by the equation of Arrhenius,[1] $\log_e \dfrac{\text{velocity at } T_2}{\text{velocity at } T_1} = \dfrac{\mu}{2}\left(\dfrac{1}{T_2} - \dfrac{1}{T_2}\right)$, the rate of locomotion of *Planaria maculata* Leidy has been studied at temperatures from 7° to 30°C. inclusive. The observations have been made while the worms were creeping over the bottom of a Petri dish (136 mm. diameter) which rested on two glass rod supports suspended from the sides of a white enameled cylindrical jar (327 mm. diameter; 250 mm. deep). The jar was filled with water, the temperature of which could be regulated by withdrawing or adding water through appropriate outlet and inlets. The surface of the water was 60 mm. above the bottom of the Petri dish. A glass rod stirrer, reaching to within 10 mm. from the bottom of the jar and rotated by a small motor, aided diffusion of the hot and cold water, so that thermal equilibrium could quickly be established. Immediately beneath the Petri dish was placed a circular cardboard of the same diameter, ruled off into 10 mm. squares, and held in place between two glass plates resting on a glass cylinder. The bottom plate was provided with two vertical glass handles reaching well up above the water level, so that the position of the cardboard could be adjusted to make its lines parallel to the direction of creeping of the worms. At the side of the jar away from the observer and slightly above were placed two 25 watt electric lamps, 150 mm. apart, with reflectors 210 mm. diameter. Over the rim of each reflector was stretched thin

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[1] See papers by Cole, 1924–25; Crozier, 1924–25, *a*, *b*; Crozier and Federighi, 1924–25, *a*, *b*, *c*; Crozier and Stier, 1924–25, *a*, *b*, *c*, *d*, 1925–26; Glaser, 1924–25, 1925–26, *a*, *b*; Hecht, 1925–26; and Orr. 1924–25, listed at the end of this paper.

brown paper to render the emergent light diffuse. The center of each paper cover was 190 mm. from the center of the Petri dish, affording an illumination of about 620 meter candles at the latter point.[2] No other light fell upon the animals. When placed on the bottom of the Petri dish at the side nearest to the lamps, the planarians were forced by their negative phototropism to creep away from the source of light and towards the observer. Being illuminated from both sides the animals usually followed a straight path. As the anterior end of the worm crossed over the successive lines on the cardboard, time was taken with a stop-watch to the nearest 0.1 second. When the worm reached the opposite side, the dish was rotated sufficiently to restore the same position relative to the lights. The animal then turned through 180° and crept again towards the observer. Only occasionally did animals creep onto the vertical wall of the dish making handling by a pipette necessary.

Observations on a single animal were begun at the lower temperatures after the water had maintained its temperature to within 0.1° for 5 minutes. If successive times for creeping 10 mm. were nearly the same, five readings were taken, but when considerable variation in the times appeared ten or more readings were recorded. The temperature of the bath was then raised 1.0° or more, a 5 minute interval for thermal adaptation was allowed, and other observations were made. This procedure was repeated until the higher temperatures had been reached. During actual observations the stirrer was stopped, to prevent any mechanical stimulation of the worm. This period of time was usually about 5 minutes, during which the temperature of the bath did not vary more than 0.1°.

The isolated experimental animals were kept in small battery jars containing tap water, two small stones, and branches of the water plant Mryiophyllum. Illumination of the jars was of low intensity and diffuse. The food of the worms consisted of all the fresh beef liver they would eat, administered not oftener than once in 2 weeks.

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[2] The intensity of the light was measured by means of a grease spot photometer using a Hefner lamp. The candle power obtained was multiplied by 0.9 to convert to international candles, and the meter candle value was calculated from the formula m.c. $= \dfrac{\text{c.p.}}{d^2}$ where d is expressed in meters.

Under these conditions the worms remained normal and healthy in every visible respect. Over a period of 3 months not a single animal died out of a total of 123.

<div align="center">II.</div>

Over 2000 observations were made on 14 animals, but not all of these could be analyzed because of variations in behavior. Five individuals consistently gave unreliable results due to the following types of behavior: (1) creeping with the anterior or posterior end of the body held at an angle above the substratum; (2) frequent crawling movements like those of earthworms (*cf.* Pearl, 1902–03, and Mast, 1903–04); (3) irregular locomotion, describing variously curved paths; and (4) frequent periods of quiescence during which the body was almost maximally contracted. All records in which any such behavior appeared were discarded. The other 9 animals gave reliable results which are presented in this paper.

Subjecting *Planaria* to different temperatures between 7° and 30°C. shows clearly that the velocity of locomotion increases with the temperature. Below 12° the body is more or less contracted, movement is sluggish, and the velocity varies at constant temperature. Up to about 21° the velocity increases gradually to its highest value, when the animal's body is stretched along its longitudinal axis to a length about 25 per cent greater than at 10°. Above 21° the locomotor rate is not always constant, and sometimes shows no increase over that at 20–22°. When an increase appears it is always smaller proportionally than between 13° and 21°. Around 30°, the worms become quiescent.

Comparison of the individual records is easily made by plotting the logarithm of the velocity of locomotion (velocity $= 100 \times$ reciprocal of the time in seconds required to traverse 10 mm.) against the reciprocal of the absolute temperature. If the points tend to form a straight line or band the equation of Arrhenius may be applied, and the value of the constant μ may be calculated from the slope of the line which best fits the points (Crozier and Federighi, 1924–25, *a*). The plots will also reveal any critical temperatures at which the value of μ suddenly changes. This treatment of the data showed that between 13° and 21° the locomotion of animals which had been from 2 to 14 days without food exhibited a fairly constant temperature character-

istic. These animals, whose records are given in Table I, will be considered first. In Fig. 1, eight individual plots from worms numbered 1, 2, 3, 6, 8, 10, and 11 have been spaced along the vertical axis to facilitate comparison. This is legitimate since it is the slope of the lines which is important for our consideration. The vertical logarithmic scale is of course identical for all the plots, the unit of distance being 0.2. The points are separate observations at different tempera-

TABLE I.

Values for μ at Intermediate Temperatures Obtained from Animals Which Had Been from 2 to 17 Days without Food.

No. of animal.	Days after feeding.	μ
1	14	11,300
2	14	11,650
3	14	11,600
6	2	10,650
6	3	10,800
6	7	11,000
6	11	10,900
8	4	11,450
8	5	11,300
9	3	10,900
10	10	11,100
11	10	10,900
Average..		11,129 ± 61
6	17	14,750
9	16	14,500
11	14	14,600
12	15	14,600
Average..		14,612 ± 30

tures. In some cases successive readings were identical, so that fewer than five points sometimes appear at one temperature. The lines have been drawn by inspection through the high and low extremes between 13° and 21°, and from the slopes of the lines the values of μ have been calculated. The critical temperatures are clearly 13° and 21° in the vicinities of which the value of μ suddenly changes. But within this region the temperature characteristic, as determined
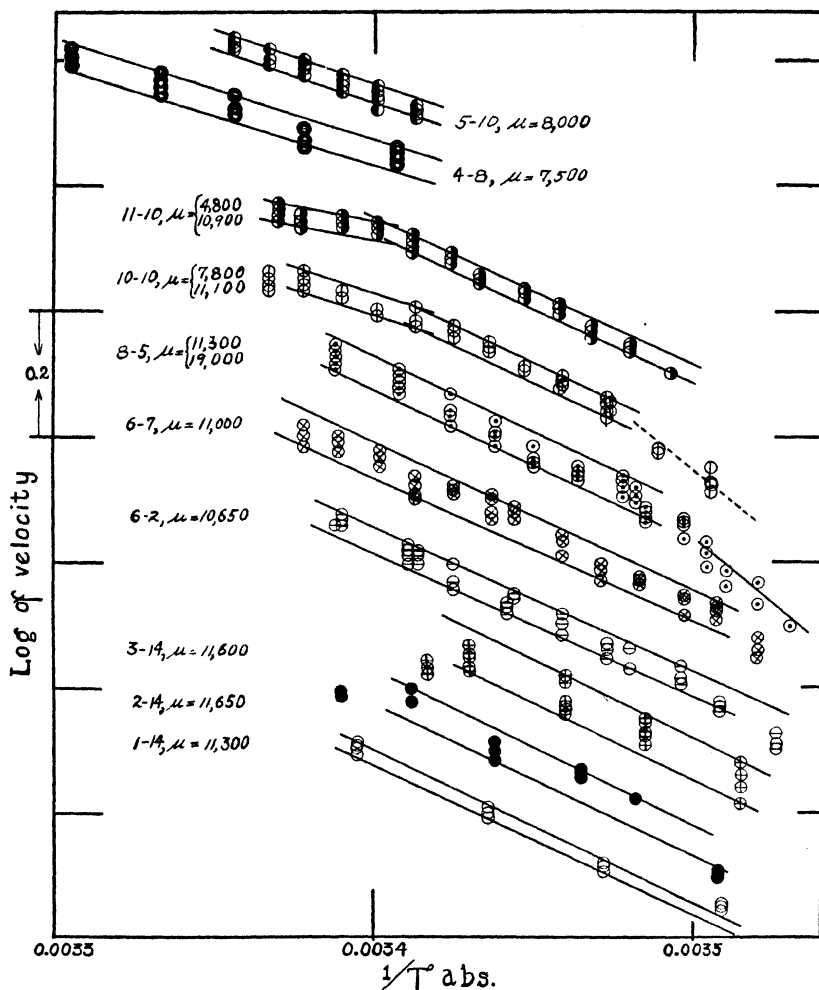
FIG. 1. Plots of observations from 10 individuals which had been from 2 to 14 days without food. The first number identifies the animal, and the second indicates the number of days after feeding. The logarithm of the velocity of locomotion is plotted against the reciprocal of the absolute temperature. Each point represents a single observation, except where two or more successive observations were identical, and the lines have been drawn by inspection through the extreme variates between 13° and 21°. The values of μ have been calculated from the slopes of the lines. For the sake of comparison, the plots have been spaced along the vertical axis with a uniform logarithmic scale the unit of which is 0.2.

by the slope of the lines, varies only from 10,650 to 11,650, the average being 11,129 with a probable error of the mean of 61. It is to be noted that this is similar to the value found in other animals where the determining reaction is supposed to be a catalyzed oxidation (*cf.* Crozier, 1924–25, *b*).

Above and below the critical temperatures regularity in the locomotor velocity disappears. Animal 8, as shown in Fig. 1, gave the best series of observations below 13°. The other worms showed consider-

TABLE II.

Values for μ at Low and High Temperatures Obtained from Animals on the Day of Feeding and on Subsequent Days.

Low temperatures.			High temperatures.		
No. of animal.	Days after feeding.	μ	No. of animal.	Days after feeding.	μ
6	$\frac{1}{6}$	18,750	4	8	7,500
6	1	19,300*	5	10	8,000
6	2	19,400	6	$\frac{1}{6}$	7,610
6	3	16,150	9	$\frac{1}{3}$	8,300
6	6	22,000*	9	2	8,700
8	4	18,000	10	10	7,800
8	5	19,000	11	10	4,800
9	$\frac{1}{8}$	14,700			
9	$1\frac{1}{4}$	17,600*	Average................		7,530
9	3	18,400*			
10	10	19,200*			
Average...................		18,409			

* Values obtained from a small number of plotted points.

able variation in their rates at constant temperature in this range, but in no case was the rate greater than at 13°. Similarly, at the higher temperatures consistently regular results were not obtained. Many worms showed no increase in rate at all above 21°, and not a few remained quiescent. A few records were obtained, however, and when plotted, the points fell fairly regularly within parallel straight lines, as shown in Fig. 1, Nos. 4, 5, 10, and 11. This condition is to be contrasted with that found at the lower temperatures, where the scattering of the plotted points prevented accurate representation by

parallel lines. In Table II appear the values of μ which were obtained from the high and low temperatures. The former are probably much more reliable than the latter, and give a mean value of 7,530. If Animal 11, which shows a value (4,800) widely differing from the others, be omitted the corrected mean becomes 7,985. This is very close to the value found by Crozier, and by Glaser (*loc. cit.*) for other kinds of activities at higher temperatures. The much higher value at low temperatures, although perhaps not so accurate, also corresponds roughly with what has been found in other processes at similar temperatures. The results obtained from the low temperatures lead to suggestions only, since they are insufficient for a complete interpretation.

It appears from these experiments that the thermal increment from about 13° to about 21° for locomotion in *Planaria* which have been without food for from 2 to 14 days, is 11,100. Below 13° it becomes much larger, showing considerable variation from 15,000 to 22,000. Above 21° the thermal increment decreases, and seems to lie between 7,500 and 8,000. In both these extreme regions the value of μ does not appear to be changed as a result of feeding as is the case at intermediate temperatures.

<center>III.</center>

Planaria which were tested soon after feeding revealed quite a different ratio of increase in locomotion at the intermediate temperatures. An attempt was made to study this modification of the thermal increment. Observations were made on animals a few hours after feeding, and frequently thereafter up to 2 days. During this period it was very difficult to secure regular and concordant results. The worms were very sluggish, showed much indefinite creeping, and frequently came to rest. Only 2 of them, Nos. 6 and 9, gave results which could be analyzed. Their records are given in Table III. The critical temperatures within 4 hours after feeding seem to be around 18° and 25°; after 24 hours they are at 15° and 25°, and after 48 hours they have shifted to 13° and 21°. At the lower temperatures μ is not much changed, but over the median range it increases from around 7,000 to near 11,000. At the higher temperatures the values are not clear, since the worms usually showed no increase in rate. These suggestions are merely tentative, in view of the small number of cases.

There can be little doubt, however, that immediately after feeding and for at least 48 hours thereafter the thermal increments for locomotion in *Planaria* are quite different at the intermediate temperatures than subsequently.   It has been reported by Willier, Hyman, and Rifenburgh (1925) that during the first few hours after feeding rather striking changes in the food and in the intestinal cells take place. These are followed by a slow digestive process which ends at about the 5th day.   Thereafter up to 6 weeks the fat and protein content as well as the histological appearance remain unchanged.   It is probable that profound metabolic changes occur soon after feeding and during digestion, and that these might shift the velocities of the reac-

TABLE III.

Changes in the Constant, μ, As Produced by Feeding.

No. of animal.	Hrs. after feeding.	High temperatures.	Median temperatures.	Low temperatures.
6	4	25–30°:3,600	18–25°: 7,600	9–18°:18,750
9	3	25–30° 0	18–26°: 8,300	13–18°:14,700
6	24	25–30° 0	15–25°: 9,800	10–15°:19,400
9	30	25–30° 0	15–25°:10,100	12–15°:17,600
6	48	25–30° 0	10–22°:10,850	
9	48	20–25°:8,700	13–20°:16,100	10–13°:18,400

tions underlying the locomotor activity so that control passes from one process to another.   After 2 days, and up to 14 days, there is no change of control, indicating perhaps a rather uniform metabolic state.

Animals 6, 9, and 11, which had previously given values for μ of about 11,000, showed an increase to 14,600 when tested 17, 16, and 14 days after feeding respectively.   No. 12, an animal not tried before, yielded a similar value (see Table I).   It appears in the three former cases that the temperature characteristic has changed again, although the reason is not clear.   It is not likely that this new value is the result of faulty technique or inaccurate observations, because the tests were performed in the same way as all the others, and when repeated on 2 of the worms gave almost identical results.   Other cases of association between temperature characteristics, 8,000 and 11,000 with an occa-

sional appearance of 14,500 have been reported by Crozier and by Glaser (*loc. cit.*). The modification of μ, as by feeding in the case of *Planaria*, coincides with the conception of experimental control of μ as advanced by Crozier and Stier (1924–25, *a*).

IV.

SUMMARY.

A consideration of the temperature characteristics or thermal increments for locomotion in *Planaria* shows that they agree essentially with those reported for certain activities of other animals (Crozier, and Glaser[1]). A process with the lowest increment (μ = 7,000 to 8,000) assumes control of the locomotor rate at temperatures above 20–22°; that with the highest increment (μ = 18,000 to 22,000) controls below 13°; and one with an intermediate value (μ = 11,100) is in command at the intermediate temperatures (13–21°). Another reaction with increment μ = 14,600 may, under certain conditions (*e.g.* 2 weeks after feeding), control the series over the median range. Excepting the latter, these increments are typical of catalyzed oxidative reactions (Crozier 1924–25, *b*) so that when these are in control it may be assumed that respiration is the fundamental process determining the rate of locomotion. Feeding produces a modification of the increment throughout the median range of temperatures, up to 2 days afterward.

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A SYNTHETIC FOOD MEDIUM FOR THE CULTIVATION OF DROSOPHILA.

PRELIMINARY NOTE.

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I.

For a number of years past the writer has been studying the duration of life of the fruit-fly, *Drosophila melanogaster*, and the factors which influence it.[1] As this work has progressed it has become more and more evident that the degree of quantitative precision desirable in experimental actuarial work was not attainable when the flies were grown upon a medium which has as its chief ingredient so variable a product, seasonally and otherwise, as the banana. *Drosophila* is now widely used as a laboratory animal, especially in genetic investigations, following the lead of Morgan and his students. It is generally cultivated upon the following medium, which originated in Morgan's laboratory.

H_2O... 500 cc.
Agar-agar.. 10 gm.
Banana pulp.. 500 gm.

Boil agar until dissolved—about 10 minutes. Mash bananas and add to agar and water and boil for 5 minutes. Bananas must be ripe but not rotten. Pour into bottles for use. Allow to cool and sprinkle lightly with yeast.

The work of Guyénot,[2] Loeb and Northrop,[3] and Baumberger[4]

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[1] See a series of papers under the general title, Experimental studies on the duration of life, *Am. Naturalist*, 1921–24, lv–lviii.

[2] Guyénot, E., *Compt. rend. Soc. biol.*, 1913, lxxiv, 97, 178, 223, 270, 332, 389, 443; *Bull. biol. France et Belgique*, 1917, li, 1.

[3] Loeb, J., and Northrop, J. H., *Proc. Nat. Acad. Sc.*, 1916, ii, 456; *J. Biol. Chem.*, 1916, xxvii, 309; 1917, xxxii, 103. Northrop, J. H., *J. Biol. Chem.*, 1917, xxxii, 123; 1917, xxx, 181.

[4] Baumberger, J. P., *J. Exp. Zool.*, 1919, xxviii, 1.

has shown that any notion that fruit in any form is in anyway neces-
sary for any biological process in *Drosophila* is not true.   Baumberger
found that the flies "can develop normally on yeast nucleoprotein,
sugars, and salts."

In the preliminary experiments undertaken to determine the essen-
tial conditions for the making of a satisfactory synthetic food medium
it was found that the acidity of any medium on which *Drosophila* is
grown increases during the life of the culture, to a point where it is
stabilized by buffering.   In the banana medium this buffering action
presumably is produced by chemical compounds and reaction prod-

TABLE I.

pH and Total Acid in Banana and Synthetic Media.

Duration of culture.	pH		Cc. N/10 NaOH equivalent to total acid per gm.	
	SA	BA	SA	BA
days				
Start.	4.1	5.3	0.30	0.24
1	3.9	5.3	0.41	0.28
2	3.5	5.15	0.71	0.29
3	3.5	4.85	0.77	0.41
4	3.5	4.7	1.24	0.67
5	3.5	4.6	0.88	1.27
6	3.5	4.8	1.05	1.66
7	3.5	4.7	1.26	2.20
8	3.5	4.8	1.68	3.33
9	3.5	4.8	1.08	2.45

ucts from the banana itself.   In the synthetic media buffer salts
were included for the purpose.

Table I shows the mean changes in pH and in total acid (expressed
as cc. N/10 NaOH) in a 9 day run of cultures on standard banana
(BA series) and one of our trial synthetic media (SA series), the com-
position of which it is not necessary to detail here, as it was subse-
quently discarded in favor of a more satisfactory medium.

The general biological results on fertility and mortality of the flies
in these experiments showed that *Drosophila* could be successfully
cultivated on an entirely artificial medium, in which no natural fruit
product was present, and which had a much higher degree of acidity,

as indicated by the pH, than has the standard banana medium in common use.   There are certain obvious practical advantages in carrying the acidity of the culture medium to as high a point as possible, having regard to normal behavior and vitality of the flies themselves, because if the pH of the medium can be brought below the limit for the growth of moulds and bacteria which occasionally contaminate cultures of *Drosophila*, the deleterious effects of these other organisms in experimental work will be automatically avoided.

On the basis of these and other experiments there was devised a synthetic medium, called in the laboratory records S-101, which has proved after exhaustive tests to be extremely satisfactory.

The composition and mode of preparation of this new synthetic medium are as follows:

Solution A.	Cane-sugar	500	gm.
	$KNaC_4H_4O_6 4H_2O$	50	"
	$(NH_4)_2SO_4$	12	"
	$MgSO_4 7H_2O$	3	"
	$CaCl_2$	1.5	"
	H_2O to make 3000 cc. of solution.		
Solution B.	Agar-agar	135	gm.
	Tartaric acid $(C_4H_6O_6)$	30	"
	KH_2PO_4	6	"
	H_2O to make 3000 cc. of solution.		

Melt the agar thoroughly in the water with heat, add the salts, and for the medium to be used in the fly bottles, mix equal parts of Solutions A and B.   For some kinds of work it has proved desirable to have the final food a little less stiff, in which case a smaller amount of agar is used, without changing the composition otherwise.

This medium has a pH when freshly made, cooled, and the agar set, of approximately 3.7.   As the flies live upon it the pH falls to a value of 3.0, or in some cases even lower.

<center>II.</center>

This new medium has been tested in a great variety of ways in the laboratory.   It has proven so satisfactory that all of our *Drosophila* stocks are now carried on it, as a routine.   On account of its high acidity there is practically never any contamination of the cultures by troublesome bacteria.   In particular *Bacillus subtilis*, which can make

a great deal of trouble on the standard banana medium, never gets a foothold on this medium S-101. Some moulds will grow on it, but the trouble from this source in routine *Drosophila* work is greatly reduced by the use of this medium.

In order to test the influence of this medium on the fertility of *Drosophila* a series of experiments was performed, according to the following plan.

Using wild type flies of a line-bred strain, half pint milk bottles were set up according to the scheme which follows:

No. of bottles.	No. of parent flies put in each bottle at start.	Initial density (flies per bottle).	Medium.
4	1 pair.	2	Two bottles at each specified density were filled to the depth of ¾ in. with standard banana medium, and lightly seeded with yeast.
4	2 pairs.	4	
4	4 "	8	
4	8 "	16	The other two bottles at each specified density were filled to the same depth with S-101 medium, lightly seeded with yeast, and incubated at room temperature 2 days before the flies were put in.
4	16 "	32	
4	32 "	64	

From these conditions it is seen that volume of food and surface area of food were the same in all bottles. Density of population in the strict sense was selected as the variable in these experiments.

All of the bottles were placed into the same incubator operating at 25°C. and carried at that temperature throughout the experiment. Each day each bottle was examined, any dead flies removed, and a record made of the date of death and the sex of the fly. At the end of 8 days the parent flies were removed from the bottles, before any of their progeny had emerged as imagoes. The bottles themselves were then continued in the incubator and counts of the progeny emerging as imagoes made each day for a period of 8 days after the first progeny fly emerged.

III.

The results respecting fertility in these experiments are set forth in Tables II and III. In these tables are recorded the initial population densities at which each bottle started (number of flies per bottle, all

bottles being the same size and containing the same volume and sur-
face area of food, the volume of air space above food); the mean den-
sity of population over the 8 day period, which figure takes into account
the number and time of the death of all the parent flies; the number of
female days, being the sum over 8 days of the number of female parent
flies in each bottle each day; the absolute number of progeny flies
produced in 8 days forward from the time of emergence of the first

TABLE II.

The Production of Drosophila melanogaster on the Synthetic Medium S-101.
Averages.

Initial density of population.	Mean density over 8 day period.	Total female days.	Total progeny in 8 days.	Progeny per female per day.	Total deaths in 8 days.	Death rate over 8 day period.
2	2.00	16	551	34.44	0	0
4	3.90	28	703	25.11	1	12.5
8	7.80	64	710	11.09	2	12.5
16	15.75	124	612	4.94	2	6.25
32	31.40	247	468	1.89	5	7.81
64	61.66	493	499	1.01	12	9.38

TABLE III.

The Production of Drosophila melanogaster on Standard Banana Medium. *Averages.*

Initial density of population.	Mean density over 8 day period.	Total female days.	Total progeny in 8 days.	Progeny per female per day.	Total deaths in 8 days.	Death rate over 8 day period.
2	2.00	16	373	23.31	0	0
4	3.65	26	380	14.62	1	12.5
8	7.65	66	435	6.59	5	31.25
16	15.60	120	353	2.94	8	25.0
32	31.00	226	263	1.16	23	35.9
64	62.53	494	250	0.55	31	24.2

progeny fly; the number of progeny produced per female per day over
the 8 day period, got by dividing the figures in the 4th column by those
in the 3rd column; the total number of deaths among the parent flies
in the 8 day period; the death rate per 100 exposed to risk over the 8
day period, got by dividing the total deaths ($\times$ 100) by the number of
flies exposed to risk of dying at the beginning of the period.

It is at once obvious from the data in Tables II and III that many

more progeny flies (imagoes) per bottle were produced on the synthetic, S-101, food than on the standard banana medium, the total volume and surface area of food being the same in the two series. This was true of all population densities. The absolute progeny productivity curves rise rather sharply from initial density 2, to a high point at initial density 8. They then fall off rapidly until the bottles of initial density 32 are reached. On the synthetic medium (S-101) the total absolute progeny produced per bottle is a little higher at initial density 64 than at initial density 32, whereas in the case of the banana series the absolute productivity value at initial density 64 is slightly lower than at initial density 32.

The same thing is shown if the more precise method of expressing fertility in terms of progeny per female per day is adopted. The relative amount of this excess is shown by the following percentage figures, which are the percentages which the differences between the two series are of the banana figures.

Percentage Increase in Fertility (Progeny Produced per Female per Day) on the Synthetic Medium S-101, as Compared with Standard Banana Medium.

Initial density.	Percentage increase.
2	47.7
4	71.8
8	68.3
16	68.0
32	62.9
64	98.0

There can be no doubt that the production of progeny, however measured, is much higher on the synthetic medium than on standard banana.

It may be noted, although it is not our purpose to discuss this point especially in this paper, that the results of these experiments agree closely with those obtained by Pearl and Parker[5] in their earlier study of the effect of density of population upon fertility in *Drosophila*, in which work standard banana medium was used.

[5] Pearl, R., and Parker, S. L., *Proc. Nat. Acad. Sc.*, 1922, viii, 212. See also Pearl, R., The biology of population growth, New York, 1925.

The difference between the two series in respect of mortality is quite as striking as that just shown in fertility. Whereas in the 8 days only 8.73 per cent died of the 252 flies exposed to risk of dying in the S-101 bottles, 26.98 per cent of the 252 flies exposed to risk over the 8 days in the banana bottles died. The mortality was relatively three times as great on the banana medium as on the synthetic. The mortality on the banana medium was heavier in this experiment than is usual in our work, so that it would be unwarranted to conclude that generally the new synthetic medium will show as great a superiority in respect of mortality as it did in this particular case. Yet, in spite of this necessary reservation, we feel reasonably certain from other experience with this new medium that there will generally be found to be a smaller mortality of the flies kept on S-101 medium than of those kept on standard banana medium. Experiments are now in progress from which we expect to be able to present much more detailed figures covering this question of relative mortality on the two media.

SUMMARY.

In this paper is described the composition and method of making a standard synthetic medium for the laboratory cultivation of *Drosophila melanogaster*.

It is shown that this medium is greatly superior to the banana medium commonly used for this purpose in respect of both the fertility and the mortality of flies kept on it. The range of superiority in respect of fertility is at different densities of population from about 48 per cent at the lowest, to 98 per cent at the highest densities experimentally reported here. The general experience of the laboratory with this medium, which frees experimental work on *Drosophila* from the incubus of the highly variable banana, shows it to have other points of superiority besides those discussed here.

A detailed account of this investigation, in which Dr. W. B. D. Penniman, Dr. Mary Gover, and Miss Agnes Allen shared, will be published elsewhere.

REVERSAL OF PHOTOTROPISM IN DIEMYCTYLUS VIRIDESCENS.

By T. J. B. STIER.

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It has been known that the newts of *Diemyctylus viridescens* are phototropic. Pearse (1909–10) states that the land form is positively phototropic; Pope (1924) and Reese (1917) give evidence that the water form is also phototropic.

When testing the phototropism of *Diemyctylus*[1] it was noted that the characteristic phototropic behavior was different when freshly collected animals were compared with those which had been kept without food in a terrarium or starved for 4 days in a glass jar. The variation of these three lots is expressed in percentages in the following table:

	No. tested.	Positive.	Negative.	Indifferent to light.
		per cent	*per cent*	*per cent*
Freshly collected..............	42	42.8	9.5	47.6
In terrarium, 2 wks..........	41	9.7	65.8	24.3
Starved in glass jar...........	9	11.1	77.7	11.1

From these results it is apparent that for freshly collected animals the positive response, or indifference to light, was characteristic; whereas for starved animals the negative reaction was predominant. The animals were started creeping under faint red illumination and after progressing about 10 cm. a beam of white light was admitted perpendicular to their path. The newts then either moved definitely away from the light, turned towards the light, or continued straight

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ahead. The trail along which the animals walked was 42 cm. from the center of the filament of a 2.5 volt, 1/4 ampere, flashlight bulb. At the observation point the intensity of the light was about 11.3 m.c. Each animal was tested but once. Pearse (1909–10) tested each animal about twenty times by placing it in a beam of light and then noting its reaction.

Thus it seemed that there was perhaps some relation between starvation and the occurrence of negative phototropism; or, to reverse the analysis, between feeding and the abolition of negative phototropism. Thinking that there might be some likeness between this case and the case of *Limax*, whose negative phototropism is temporarily suppressed by certain types of feeding (Crozier and Libby, 1925), *Diemyctylus* which had been tested for phototropism were dissected and the contents of the alimentary tract examined.

In the course of the summer the stomach contents of over 40 tested animals were examined. Records showed that 80 per cent of all the positively phototropic animals had full stomachs, and that 100 per cent of the negatively phototropic animals had the alimentary tract empty. However, in animals which were indifferent to light the stomach contents were either in a complete state of digestion and the intestine was full; or the stomach was empty and the intestine and cloaca showed evidence of recent feeding.

The dissections therefore clearly indicated that an empty alimentary tract accompanied a state of negative phototropic behavior, and that presence of food in the stomach was correlated with positive phototropism.

In order to further test this relationship, 19 animals were starved in glass containers for 5 days. After being dark-adapted for 2 hours they were tested for phototropic reaction. They gave the result characteristic for starved *Diemyctylus*—68 per cent were negative, 10 per cent positive, and 22 per cent were indifferent.

Each animal which gave the negative reaction was then fed two larvæ of a common red ant. The larvæ were put into the esophagus with forceps. It had been observed while dissecting the newts that ant larvæ made up the greater bulk of their food. After 1 hour in the dark room these fed newts were again tested—33 per cent were negative and 66 per cent were indifferent. When they were tested 3 hours after feeding, however, all were indifferent to light.

SUMMARY.

In its response to light of approximately 11.3 m.c. intensity *Diemyctylus viridescens* may be either positive or negative. Negative phototropism is characteristic of animals from which food has been withheld. Feeding suppresses this negative phototropism.

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NOTE ON THE DISTRIBUTION OF CRITICAL TEMPERATURES FOR BIOLOGICAL PROCESSES.

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I.

A survey of data on the variation in velocities or frequencies of vital activities as controlled by temperature shows that in general there obtain rectilinear relationships between logarithm of velocity (or frequency) and reciprocal of absolute temperature. The constant E or μ, critical increment or *temperature characteristic*, which is defined by the slope of this relationship does not vary at random. Its values as determined from many series of observations upon diverse processes fall sharply into a small number of classes (Crozier, 1924). These classes must be presumed to correspond to real and distinct types of events in living matter. It has been suggested that they may in many cases be conceived to represent specific catalyzed reactions, which might thus become identifiable (Crozier, 1924, 1924–25, *a*).

Since it happens frequently, but not always, that two temperature characteristics apply to different portions of the temperature range, it is necessary to picture some form of interconnection between the corresponding processes. In part upon obvious general grounds, but also because of detailed evidence derived from study of a phenomenon for which no other interpretation seemed possible (Crozier and Federighi, 1923–24), it was assumed that the relationship might frequently be a catenary one, although certain other possibilities are by no means excluded (Crozier, 1924–25, *b*). From this standpoint one conceives, as determining the frequency of heart beat, for example, a series of rapid, linked, reactions, the velocity of each determined by the specific velocity of formation of its proper catalyst. The controlling step in such a series might then differ in two ranges of temperature according to the diverse effects of temperature upon the magnitudes of the several

velocity constants.   This view affords a rational interpretation of the fact that, in suitable cases, experimental control of the temperature characteristic exhibited permits the uncovering, so to speak, of processes known to be allied but ordinarily concealed (Crozier and Stier, 1924-25, *a* and other series of experiments as yet unpublished).

Critical temperatures, defined as regions in which control changes from one underlying reaction to another, have been determined from the points of intersection of lines fitting portions of the temperature-velocity graphs (Crozier, 1924–25, *a*).   But this is not the only type of critical temperature which must be recognized.   In the respiratory rhythm of anurans (Crozier and Stier, 1924–25, *b*) there is discovered a type of break in the temperature graph which results from a change of frequency without change of temperature characteristic.   This is best understood as due to a change in *amount* of the corresponding catalyst.   It was pointed out (Crozier and Stier, 1924–25, *b*) that this type of effect, which occurs in several other well defined instances, and which is found to be subject to experimental control, presumably depends upon physical alteration of the catalytic material.

To these categories of critical temperatures there may be added temperatures (1) at which trigger effects (Crozier and Stier, 1924–25, *a*) appear; (2) at which sharp changes in behavior are apparent (Crozier and Federighi, 1924–25); and (3) at which phenomena cease to obey the rectilinear relationship between log velocity and $1/T°$abs.   The temperatures referred to under (3) represent high and low points beyond which the effect of temperature is only very slowly reversible; as a rule these points can be determined with fair precision.

The justification for putting together critical temperatures of the several classes enumerated is derived simply from the finding that the actual temperatures which clearly appear as most frequently critical in the different categories are as a matter of fact identical.

. It thus becomes of interest to discover what regularities may be found in the occurrence of critical temperatures.   Merely to refer such events to colloidal changes, as is sometimes attempted, usually explains nothing and serves merely as an apology for obscure thinking.   We desire to know why the most frequently occurring critical temperatures have certain definite values, since this information should give clews as to the mechanisms of the critical effects.

The frequency of occurrence of critical breaks, as determined by examination of a large mass of relevant published material and of a number of investigations as yet undescribed, is exhibited in Fig. 1.

This summary is published for the sake of its probable utility in connection with related discussions. To present in detail the numerous series of observations analyzed in its preparation would extend this note to unreasonable dimensions, for the literature concerned with temperature effects is very large.
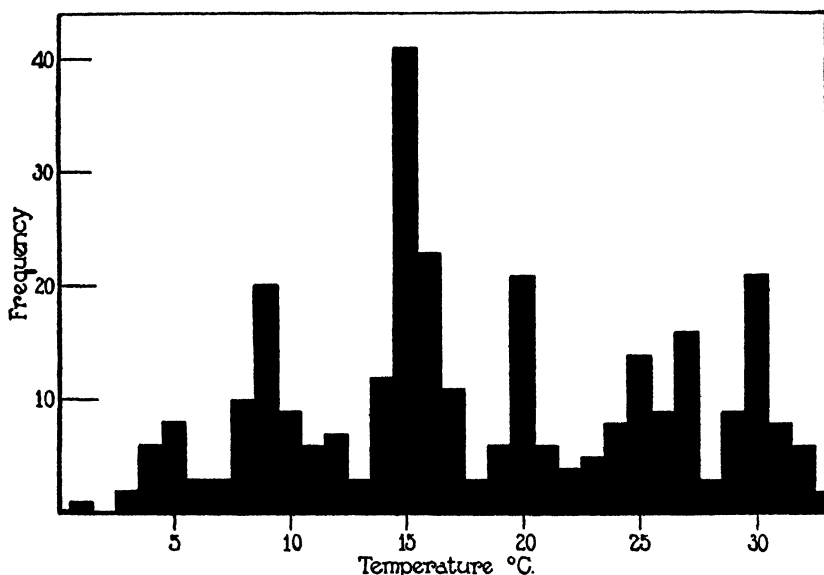
FIG. 1. Distribution of critical temperatures.

II.

It is to be expected that a collection of random critical temperatures from a variety of sources should demonstrate the more frequent occurrence of those critical temperature regions which are in a real sense properties of protoplasm in general. Despite imperfections known to be present in the basic data, it is a fair conclusion that this expectation is met by the curve in Fig. 1. The graph rises to well defined modes. These do not occur at points exactly 5° apart, nor at 5°, 10°, . . . , as they might if unfairly weighted and distributed

through a tendency of observers to work at temperatures so spaced. Moreover, a large number of the points are determined by the intersections of lines taken to provide for the same activity different values of μ for different temperature ranges, and are thus independent of the particular temperatures at which observations were made. These latter cases by themselves yield modal critical temperatures identical with those evident in Fig. 1.

For the purpose of this summary the observed critical temperatures have been rounded off to the nearest degree. Results from independent series of readings by one observer upon the temperature relations of a single sort of activity (*e. g.* heart rate) in one kind of organism have been averaged. This procedure is legitimate, for in addition to uncertainties of observation it should be stated that in cases which have been most extensively studied, with homogeneous material, there is evidence of real but not extensive variation in critical temperature. Again, instances are available in which a critical temperature, as here defined, may be shifted under experimental treatment; these latter cases have been excluded from the present summary.

The individual entries contributed to Fig. 1 differ greatly in weight and precision of observational basis. The phenomena considered are very diverse, from growth of fungi to minimal critical temperatures for the hearts of mammals. The total number of cases is sufficiently large, however, namely 286[1] to give real opportunity for manifestation of any significant tendency to form a unimodal frequency distribution. The positions of the peaks are not materially modified by other methods of grouping, and correspond with the positions of the critical temperatures as obtained from those series of observations judged on independent grounds to be qualitatively best. Where the numbers of instances begin to be adequate, each mode represents the peak of a frequency distribution.

The temperatures which may be given as the most frequently occurring critical temperatures, on the basis of Fig. 1, are: 4.5°, 9°, 15°, 20°, 25°, 27°, 30°. It is noteworthy that the frequency of occurrence is about the same for each of these points, except that at 4.5°, which is

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[1] Above 30° the available data become very difficult to interpret, because with tissues of many forms destructive effects in which the time of exposure is important are then commonly evidenced.

low, and that for 15°, which is twice as great.[2]   The occurrence of a critical region in the neighborhood of 4° to 5° gives a very interesting suggestion for the further study of these points, both as to the meaning of the critical temperatures and of their distribution.   This must for the present remain open.

III.

SUMMARY.

The critical temperatures at which irregularities appear in the relations between vital processes and temperature are not distributed at random.   As based upon detailed knowledge of individual cases, and as derived from inspection of the frequencies of occurrence, these critical points are usually found to be in the neighborhood of 4.5°, 9°, 15°, 20°, 25°, 27°, 30°.

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[2] There is enforced by this finding the conception of critical temperatures as defining thermal zones within which particular reactions occurring in protoplasm are enabled to control the activities of the organism.   Such limiting temperatures are clearly apparent in growth phenomena (Crozier, 1924–25, *b;* Bliss, 1925–26). The observations of Setchell (1915, 1920, 1925) have led him to a quite independent deduction of critical temperatures for the distribution of marine algæ and other plants, which he has been able to trace to the thermal control of growth and fructification.   The remarkable fact emerges that the critical temperatures assigned by Setchell are: 5°, 10°, 15°, 20°, and 25°.   It is scarcely possible to regard the correspondence between Setchell's results and those of the present paper as accidental.

THE DISTRIBUTION OF TEMPERATURE CHARACTERISTICS FOR BIOLOGICAL PROCESSES; CRITICAL INCREMENTS FOR HEART RATES.

By W. J. CROZIER.

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I.

Observations illustrating the changes in velocities or frequencies of vital activities with alteration of temperature are sufficiently numerous to permit the demonstration that temperature characteristics deduced for these data fall into definite classes (Crozier, 1924). Proof of this point is important for the conception that temperature characteristics may be used for the recognition of specific controlling processes or reactions in living matter. The chemical identification of these processes may thus be made possible, but this undertaking must be very carefully approached. The term "temperature characteristic" was deliberately introduced (Crozier, 1924–25, *a*) to provide a name for the quantity E, or μ, in the van't Hoff-Arrhenius equation, which should be free from the connotation of any particular view as to the mechanism of "activation." With this understanding the name "critical increment" may, however, still be employed as a convenient alternative, for it has long been recognized that the magnitudes of the temperature coefficients of biological processes as a rule correspond to those for common chemical reaction velocities. It is not to be supposed that the pursuit of this view requires denial that the temperature characteristics as measured may in some instances be referable to heats of reaction, for the descriptive equation for reaction velocity (Arrhenius) and the van't Hoff isochore are of course identical in form; in fact the formal relationship $m = m_0 \exp(-A/T)$ may hold with some precision for a variety of phenomena. But it may be pointed out at once that if in measuring the relations between velocities of vital processes and temperature one really had to do predominantly

or even frequently with shifts of mass action equilibria, then it would be reasonable to expect the quite frequent occurrence of values of E which would correspond with heats of neutralization and the like. In point of fact, however, the magnitudes 13,000, 14,000, and 15,000 cal. turn out to be strikingly infrequent (Fig. 1).

If it were to be supposed that the relationships between velocities of vital processes and temperature are really incapable of being used for purposes of identifying controlling reactions, but on the contrary are obscurely influenced by protoplasmic fluidity and other factors (cf. Snyder, 1911; Matisse, 1921), then it would be inexplicable that the same values of temperature characteristics should be obtained for homologous processes in diverse organisms (Crozier, 1924–25, a, b; Crozier and Stier, 1924–25, a, c, d; Glaser, 1924–25, 1925–26);[1] the experimental interconversion of temperature characteristics (Crozier and Stier, 1924–25, a; 1925–26) would be senseless; and there would, indeed, be no reason assignable for the fact that the Arrhenius-Marcelin-J. Rice equation does adequately describe, in the overwhelming majority of cases, the connection between velocity and temperature.

Aside from the analysis of adequate data relating to suitable phenomena, there is another method of testing the hypothesis of recurrent, discrete, recognizable, temperature characteristics. On the view that these quantities are easily modified to various degrees by differ-

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[1] The attempt to introduce considerations of protoplasmic fluidity, presumably as influencing diffusion, requires a theory of the general control of organic activities by the whole body of the cell rather than at surfaces. This is unnecessary, and at present inadmissible. When dealing with such processes as muscle relaxation (Fulton, 1925) and ameboid progression it may be necessary, for other reasons, however, to reckon with protoplasmic consistency. The serious obstacle arises that cytoplasmic viscosity of different cells (e.g. eggs of Cummingia and of Nereis) varies with temperature in totally unlike ways (cf. Weber, 1916; Heilbrunn, 1924; Pantin, 1924); there is no evidence of corresponding variations in temperature characteristics. Several writers (Snyder, 1911; Pantin, 1924) have sought to "correct" temperature coefficients for viscosity effects, under the delusion that the Q_{10} ratio should be constant—a performance for which there is no justification.

That physical changes may be important for the production of certain types of alteration in the log $V - 1/T$ graph has been expressly recognized (Crozier and Stier, 1924–25, d). But these changes may be of a relatively clear-cut character, and their interpretation is not expected to present insuperable difficulty.

ent influences, a frequency polygon of the values deduced from numerous sets of observations should take the form of a relatively simple frequency distribution. The actual polygon so obtained (Fig. 1) is definitely multimodal.

II.

For the compilation of Fig. 1 use has been made of a large number of series of observations, in greater part derived from published material[2] but including also a number of unpublished series. By a *series* is meant a set of data secured by one observer, pertaining to a given process, in a given organism. Such series are quite obviously of uneven value. Inherent differences are connected with the types of processes considered. Thus there is reason to expect measurements of growth, for example, to involve phenomena which need not be disturbing for the study of rapidly transpiring events such as the succession of heart beats; these require separate analysis. Individual series differ greatly in the numbers of observations, and in the numbers of organisms employed, as well as in the suitability of the experimental arrangements and in the accuracy, the range, and the spacing of the working temperatures. Nevertheless it has been sought to include all known available series, excluding only those in which the experimental temperatures are very few (2 or 3) and widely separated, since the possible occurrence of breaks (Crozier, 1924–25, a; Crozier and Federighi, 1923–24) at intermediate temperatures may render such observations of very doubtful significance. The sharpness of the separation of the modes in Fig. 1 is much increased if one tries to select those phenomena which seem *a priori* most likely to be suitable for significant measurement.

The method of computation has been illustrated in former papers, and is apparent in the accompanying figures. The various sets of data have been separately plotted at least three times, with different values of the coordinate units, and the best fitting lines satisfying the relation *log velocity* $= - K \ exp \ (\ \mu \ /RT)$ have been used to calculate

[2] See Kanitz (1915; 1923); Przibram (1923); and papers in *J. Gen. Physiol.*, 1924–25, 1925–26, vols. vii, viii, ix.
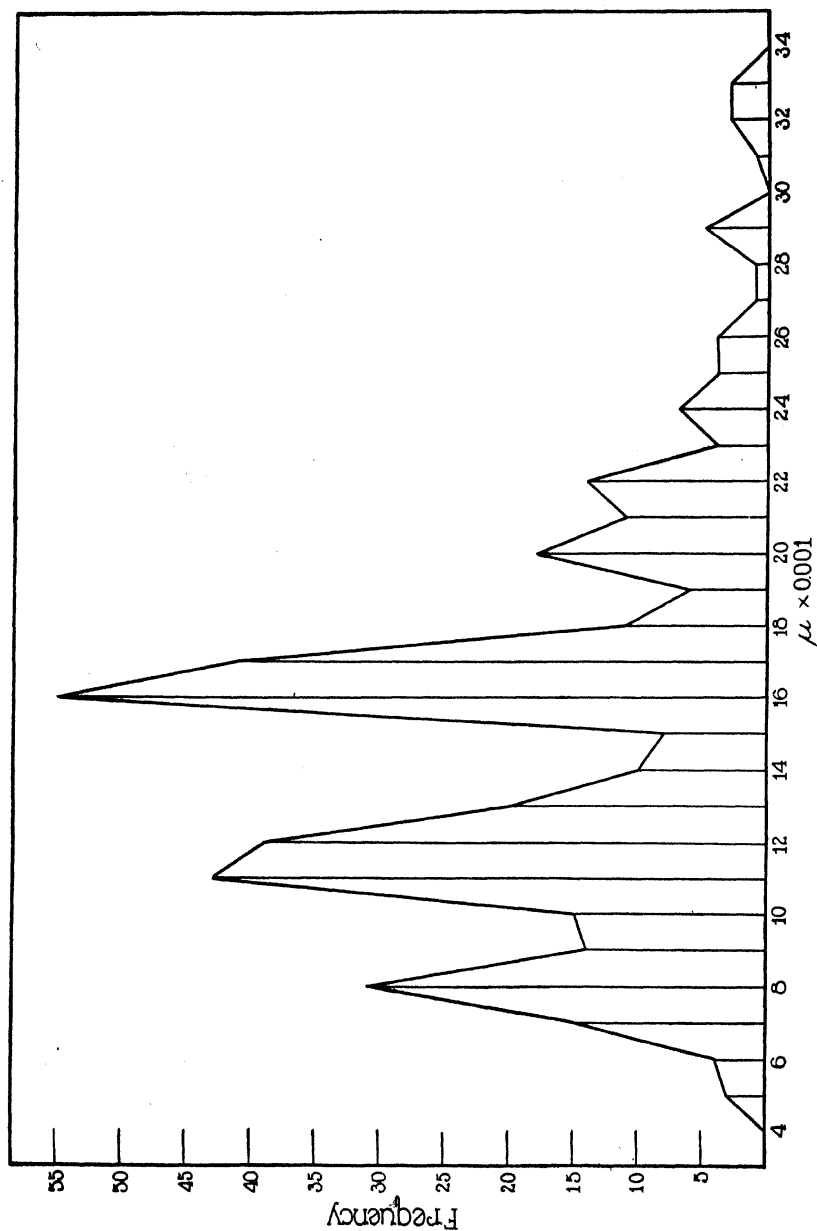
FIG. 1. Frequency of occurrence of values of μ in series of data pertaining to diverse biological phenomena.   See text.

μ.[3] The values thus independently obtained have checked to within a degree which does not alter the positions of the entries in Fig. 1.

The diversities of organisms, of technical precision, and of intrinsic weights of observations involved in the data giving rise to Fig. 1 provide every opportunity for the operation of statistical factors of chance. The non-existence of discrete, recurrent, magnitudes of μ should therefore be expected to produce in Fig. 1 a single moded frequency distribution. The most reasonable interpretation of the apparent facts, on the other hand, accepts the view that in living matter of various sorts there frequently recur certain processes, manifest as controlling processes, which are similar to the extent that they exhibit sensibly identical temperature characteristics. It should be noted that the peaks in Fig. 1 agree perfectly with magnitudes of μ which seem to rest upon the most solid observational basis.

The most probable values of these critical increments cannot be ascertained from the graph in Fig. 1. Thus there is evidence that the frequently obtained values 11,000+ and 12,000+ are organically distinct; similar consideration holds for 16,000+ and 18,000+. In the region of higher magnitudes (20,000 and above) the number of instances is insufficient, but there are available unexceptionable individual series of data yielding, for example, $\mu = 23,500$, $\mu = 32,000$. The most probable values of the characteristics typically encountered are not required for the present discussion, which aims merely to demonstrate the repeated occurrence of recognizably discrete magnitudes. This does not involve the assertion that the value of a temperature characteristic must be regarded as unmodifiable. If real fluctuation does occur, it is in Fig. 1 massed with deviations due to inadequacies of observation. The problem of modification of critical increment is one of peculiar fascination and great possibilities, as I have already intimated (Crozier, 1924-25, b), but its discussion must await further experimentation.

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[3] By proper choice of abscissæ the calculation is reduced to one setting of the slide rule. This method is much better than reliance upon a nomogram, since it is possible in fitting the lines to give weight to the latitude of variation (Crozier and Federighi, 1924-25; 1925).
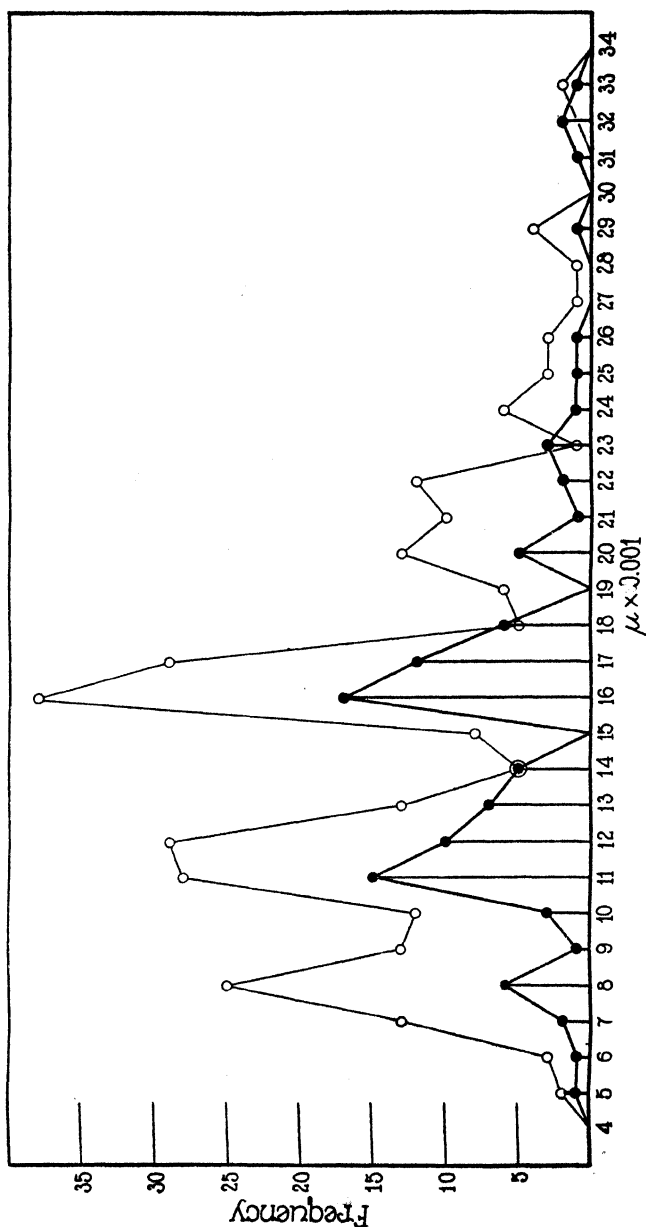
FIG. 2. Comparative distribution and frequencies of temperature characteristics obtained from (A) data pertaining to heart activities (solid black circles), and (B) all non-heart observations (white circles). As explained in the text, it is probable that many series of observations are in need of revision; this is due in part to the fact that with isolated hearts time may be a significant variable.

III.

The heart rate is easily observed in many animals, and numerous sets of data giving its variation with temperature have been published. It is proposed to illustrate with these data, and with the results from some new experiments, one further aspect of the probable occurrence in living matter of a system of processes which, in one or another case, control the change in rates of vital phenomena as determined by
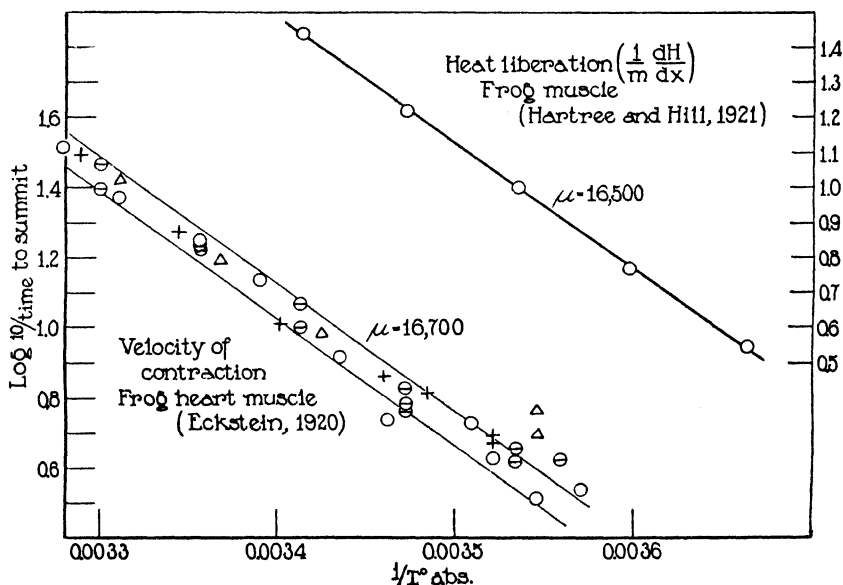
FIG. 3. Heat liberation per second of stimulation, frog skeletal muscle (Hartree and Hill, 1921).

Velocity of contraction to maximum, frog heart muscle (Eckstein, 1920). Different preparations distinguished by symbols. At very low temperatures, irregularities appear, as is not uncommon.

temperature alterations. Heart rate observations are particularly suitable because of the infrequent occurrence of breaks at intermediate temperatures. Fig. 2 shows the frequency distribution of temperature characteristics for heart activities (A), and enables its modes to be compared with those in the curve (B) for values of μ derived from all other (non-heart) sets of observations entering into Fig. 1. Again the

probable significance of the polygon A can be enhanced if one were to omit certain dubious instances.
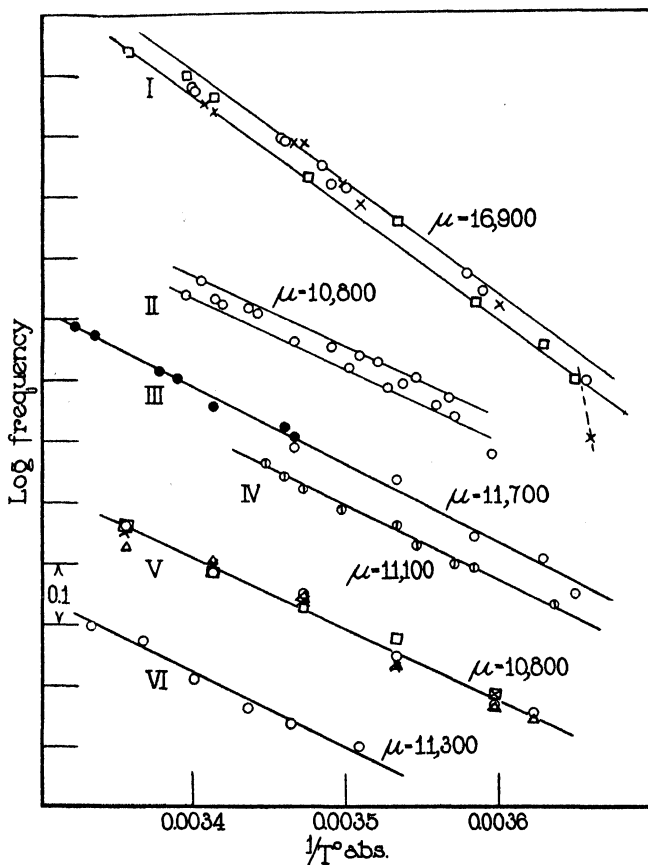
FIG. 4. Observations on frog hearts.   Data from:
I. Clark (1920–21), heart perfused with oxygenated Ringer solution ($\times$); with alkaline Ringer solution ($\bigcirc$).   Snyder (1907), ($\square$).
II. Personal observations, one series.
III. Snyder (1907), two series.
IV. Gellhorn (1924).
V. Ishihama (1924), latency in contraction of sinus ($\bigcirc$) and of ventricle ($\times$).
VI. Burdon-Sanderson and Page (1879–80), refractory period.
In I, III, and V series not significantly different have been combined by multiplication of one by a factor; each has its own symbol.

It is obvious that the two distributions are similar; their modes are coincident.[4] Therefore, the processes which in different cases control the frequency of the heart beat are similar in kind and in variety
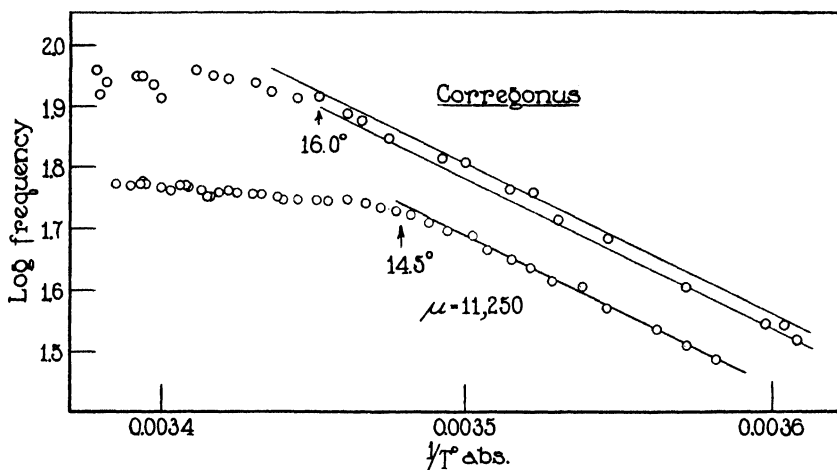
FIG. 5. Observations on heart beat frequency in two similar embryos of the lake white fish *Corregonus* (Hubbs and Crozier in 1919–20). Development had been undergone at low temperature. A temperature of 14–16° produces a frequency of contraction which is very little exceeded at higher temperatures. But the break comes at different temperatures in the two cases. Attempting to average observations, at each of a number of temperatures, from several individuals, would in the presence of such differences be very likely to produce a curvilinear relation between *log frequency* and $1/T$. In the event that breaks of different sort are present in some individuals but not in others, averaging leads to additional difficulties.

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[4] The experiments of Knowlton and Starling (1912) have been sometimes appealed to as giving evidence against the existence of a "temperature coefficient" for heart beat frequency. Knowlton and Starling (*loc. cit.*) give data for two experiments only, one series of readings with one dog heart and one with one cat heart; the perfusion pressure was varied, and the reflection of this is superimposed upon the temperature effect; in the range 37.5–27°C. the critical increment is 11,300, as found in some other cases; below 27° the alterations in arterial pressure obscure the results, which, however, are not inconsistent with the conclusion that the increment is really about 17,000. The deduction is permissible that under the conditions of their experiments (*i.e.* heart-lung preparation) the velocity of an oxidative process (Crozier, 1924–25, *b*) controls the frequency of heart beat.

to those governing the velocities of many other sorts of cellular phenomena.

Since it is legitimate to consider that the frequency of heart beat is in many cases dependent upon properties of cardiac muscle (for some recent evidence, *cf.* Copenhaver, 1925), I have not hesitated to put together in Fig. 2 increments referring to heart rates of intact animals, of isolated hearts, and to properties of cardiac muscle. It is of interest to notice that the velocity of contraction of frog heart muscle (Eckstein, 1920 (Fig. 3)) provides an increment indentical with that
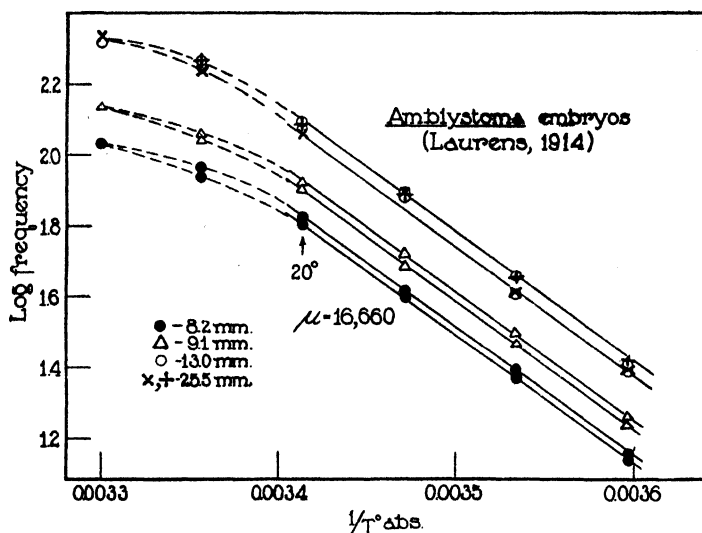
FIG. 6. The frequency of heart beat in *Amblystoma* embryos increases with size of animal, but the increment is unchanged. Above 20° thermal "destruction" occurs, so that there is failure to recover the initial rates on cooling (from Laurens, 1914).

for heat liberation (per gm. per second during stimulation) of frog skeletal muscle (Hartree and Hill, 1921). The refractory period of frog heart (Burdon-Sanderson and Page, 1879–80 (Fig. 4)) and the muscular latency (Ishihama, 1924 (Fig. 4)) show an increment which does not differ from that for latency in frog gastrocnemius (Burnett, 1906–07, $\mu = 10{,}800$; also data of Woolley, 1900, in Crozier, 1924–25, *b*); the increment for latency in smooth muscle seems defi-

nitely different (data of Viale, 1921, on stomach rings of winter frogs, give μ = 13,000 +; of Schultze, 1897, 12,200; of Stewart, 1900–01, on cat's bladder, 17,000).

Complete presentation of the material summarized in Figs. 1 and 2 is unnecessary. The heart rate data illustrate so well the kinds of relations observed that a fairly complete representation of instances for the hearts of amphibians, reptiles, and mammals is given in Figs. 4, 9, 10, and 11. (A few cases illustrated in previous papers are omitted.)
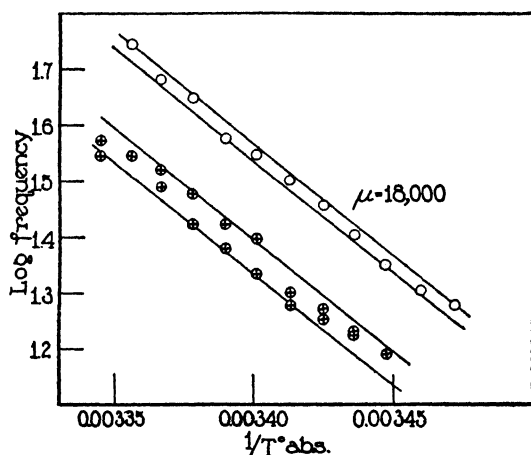
FIG. 7. The frequency of frog heart beat may be increased without sensible change of increment; lower plot, perfused with Ringer solution; upper, with Ringer plus horse serum plus adrenalin 1:500,000 (from Gellhorn, 1924).

A difficulty which arises in the interpretation of many series of published observations is due to the presentation of *average* figures derived from several individuals supposed to be similar. One sort of confusion which thus becomes possible will be understood from Fig. 5. The most significant values of μ are obtained from numerous observations upon one object.

It is clear that the same magnitudes of μ are repeatedly encountered. The μ may be constant even when the frequency is increased during growth (Fig. 6) or through alteration of the perfusing fluid (Fig. 7).

But the same kind of isolated heart may show different values for μ, quite definitely distinct, apart from deliberate experimental interference (see Fig. 8). This has a direct bearing upon the interpretation of attempts to alter the temperature coefficient of heart activity by chemical means.[5]
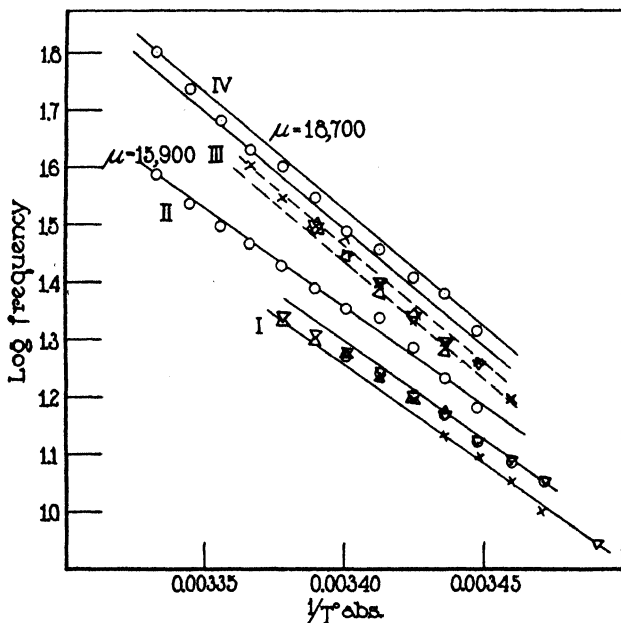
FIG. 8. The value of μ for frog heart beat frequency may differ independently of deliberate experimental treatment; I and III, perfused with Ringer solution plus horse serum; II, Ringer solution; IV, Ringer and tyramin (data from Gellhorn, 1924).

Nevertheless it is clear that the temperature characteristics for heart processes are of the same categories, quantitatively, throughout the vertebrate series (Figs. 4, 9, 10). The increments obtained with one type of heart (e.g. amphibian (Fig. 4)) are of the same classes as those encountered in other types. The occurrence of several definite μ for the same

[5] Cf. Clark (1920–21); Bouckaert, Bouckaert, and Noyons (1922); Sollmann, Mendenhall, and Stingel (1914–15).

activity, even in the same individual (*cf.* Crozier and Stier, 1924–25, *a*), is fully consistent with the idea that the control of vital processes depends, not upon a single sort of process, but upon a nexus of inter-
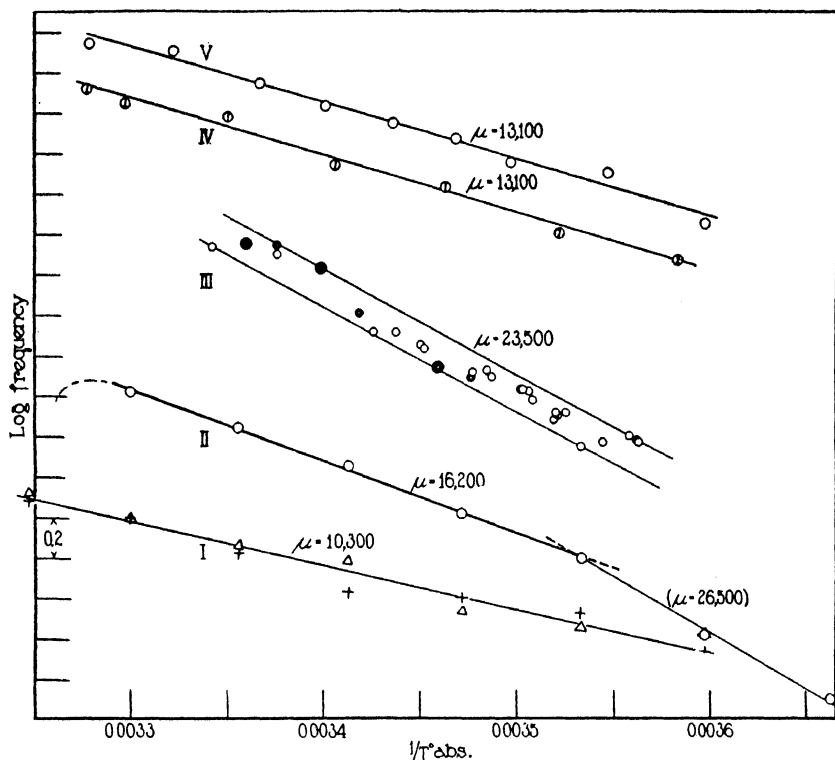
FIG. 9. I. Latency, turtle ventricle (+) and auricle (Δ) (Ishihama, 1924).

II. Latency, turtle ventricle muscle (Snyder, 1911).

III. Frequency of heart beat in a garter snake, the heart protruding through a slit in the body wall; circulation intact. Observations by Mr. T. J. B. Stier.

IV. Frequency, terrapin heart, excised (Snyder, 1905).

V. As in IV, personal observations.

related chemical reactions, constantly proceeding but with individu-ally variable rates. The examination of all available information leads to the suspicion that this complex system of controlling reactions

is ,in certain essential details constant, in living matter of whatever kind.[6]
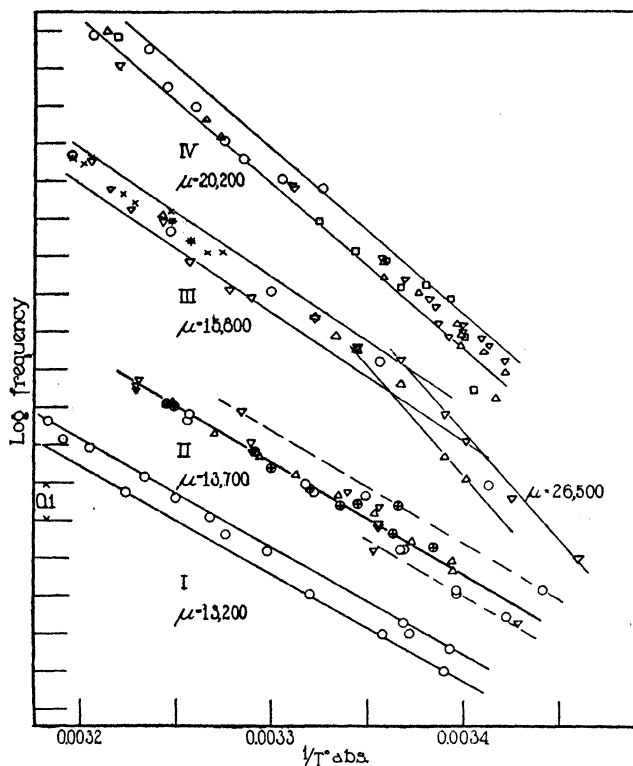
FIG. 10. I. Dog heart (Snyder, 1913).

II. Dog hearts (Frank, 1907).

III. Cat hearts (Δ, ∇) (Langendorff, 1897). Nodal strips, rabbit heart (Moorehouse, 1913).

IV. Rabbit auricle in oxygenated Ringer (Clark, 1920–21).

V. Dog heart (○) (Langendorff and Lehmann, 1906). Dog hearts (□, Δ, ∇) (Frank, 1907).

Series yielding independently temperature characteristics which do not differ significantly have been brought into close association by the use of individual factors.

[6] The data upon velocities of respiratory process, it may be stated, fully support this conception, for a good number of instances may now be added to those cited of an earlier paper (Crozier, 1924–25, *b*) in which concordant values of μ are evident.

IV.

SUMMARY.

Disregarding sources of variation known to be present in the unselected data, it is shown that the frequency distribution of temperature characteristics (critical increments) calculated from all known series of observations, pertaining to a great variety of vital processes, exhibits a number of discrete modes. This leads to the view, already derived from evidence of a more specific sort, that such critical increments may be used to characterize definite processes in a controlling system of reactions which seems to be of widespread occurrence in living matter.

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ON THE MODIFICATION OF TEMPERATURE
CHARACTERISTICS.*

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I.

An endeavor to find if specific meanings might be attached to the temperature characteristics of vital processes has led to the view[1] that common activities of organisms appear to be governed by the velocities of members of a system of chemical reactions, identified to the extent that they yield quantitatively concordant critical increments, which are repeatedly encountered in very different kinds of living matter (Crozier, 1925–26). Experimentally determining which of several possible critical increments a given phenomenon shall be made to exhibit thus signifies control of specific governing reactions (Crozier and Stier, 1924–25, *a*), and makes possible, even without great regard for the theory giving rise to it, a new type of analysis. Its development is facilitated by knowledge of instances in which it is possible to modify the temperature characteristic, even when no truly reversible determination may be attained.

Several quite different possible kinds of modification can be predicted to occur, on the basis of what is already known of the temperature relations. It will be shown that some of these possibilities are realizable. They may be roughly enumerated:

(1) The velocity or the latitude of variation may be changed without change of increment; (2) a normal temperature characteristic

may be slightly increased, or decreased; (3) it may be changed to a quite different value, either obviously composite[2] or numerically equivalent to one already familiar in other essentially homologous processes; (4) a rectilinear relation between *log velocity* and $1/T\ K$ may be changed to a curve; (5) a break in the temperature graph, with two critical increments over two ranges of temperature, may be
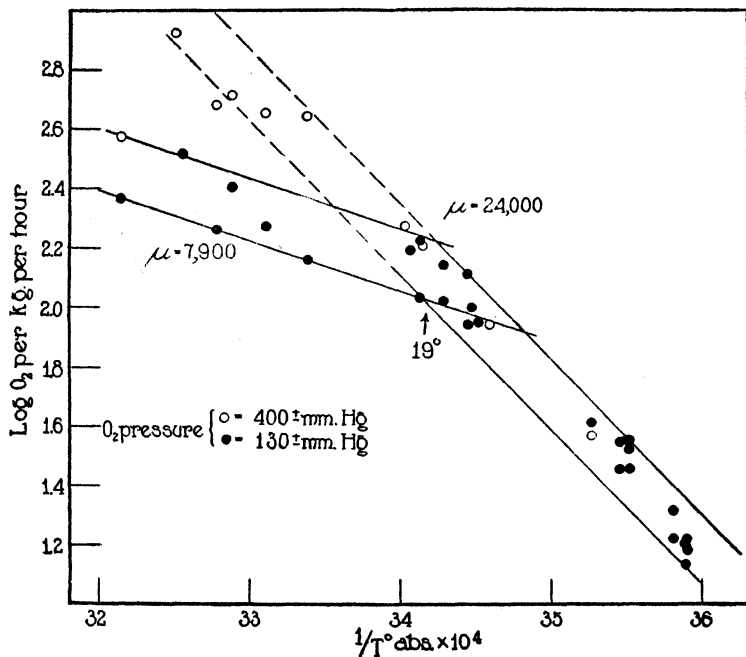
FIG. 1. At low oxygen pressures, the temperature characteristics for velocity of oxygen usage by leeches are $\mu = 7,900$ and $\mu = 24,000$. When the oxygen pressure is increased the break is obliterated. Data from Pütter (1914).

obliterated; or one may be caused to appear in a previously unbroken line; (6) a break of the sort described for breathing rhythm of anurans

[2] Thus when it was sought to study the temperature coefficients for the action of digitalis alkaloids upon the heart (frog; Sollmann, Mendenhall, and Stingel, 1914–15) the Q_{10} ratio was found to be very high. The value of μ in the Arrhenius equation which fairly well fits these data is about 40,000. This seems due to the fact that the activity of the heart, as well as the temperature, controls the speed of the toxic action.
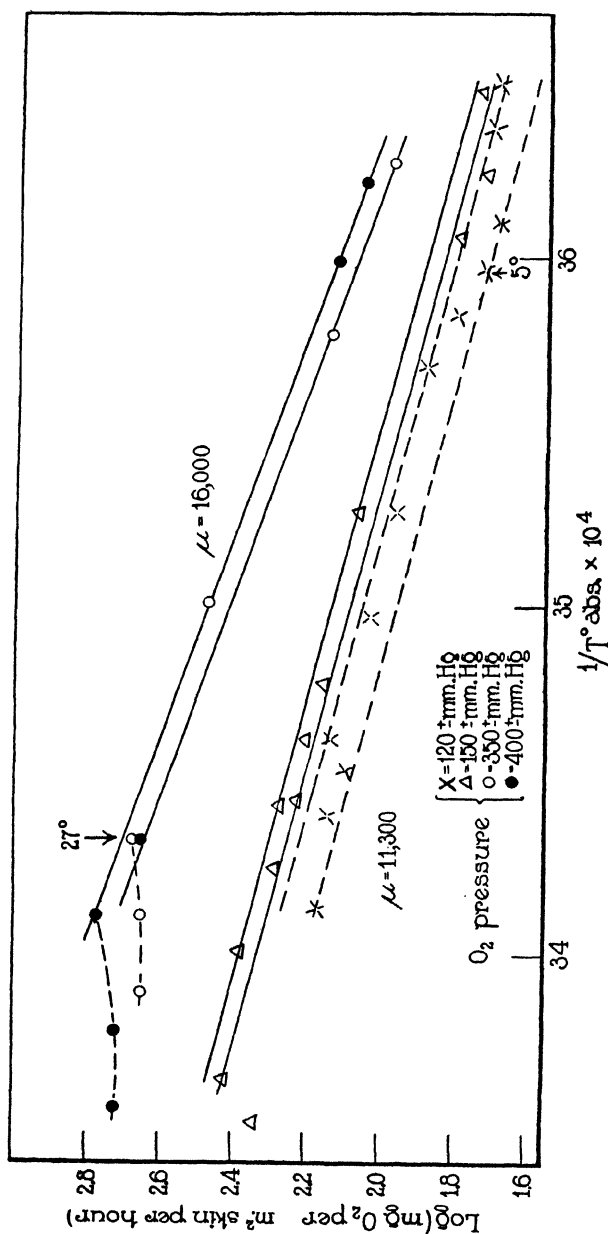
FIG. 2. Variation in μ for velocity of oxygen utilization (via skin) in frogs at different temperatures, when the oxygen pressure is changed. Data from Pütter (1914). The increments fairly assignable belong to the two sorts commonly found for respiration. (Below 5° the observations at 350 mm. O_2 pressure are irregular.)

(Crozier and Stier, 1924–25, *b*), signifying change of velocity without change of increment, may be obliterated; or one may be developed; (7) the type of change mentioned in (6) might be combined with a change of increment.

Alterations of types (1), (3), (4), and (5) are already known. Change of type (4) is readily obtained if due time for recovery is not allowed following exposure of the organism to very high or to very low temperature, and in certain types of toxic action (*cf.* Crozier, 1924–25). In addition to instances given in previous papers[1] certain other illustrations taken from cases in the literature of temperature coefficients may be cited here (Figs. 1, 2); they do not exhaust those available.

We desire to record some new instances of this sort, calling attention particularly to the fact that when change of increment is induced by experiment the new value is in each case that of an increment already known to be of frequent occurrence and known to be related to, or in some way organically connected with, the initial value. In doing this we give details of single experiments, that is records pertaining to individual organisms, without describing similar confirmatory instances. Experience shows that it is most important in such work to obtain numerous measurements upon single organisms.

We also describe, in greater detail, an experiment of more definite kind dealing with reversible control of the increment for heart beat frequency in *Limax*.

<div align="center">II.</div>

The temperature characteristics for frequency of pharyngeal breathing movements of frogs is of the order $\mu = 8,500\pm$ (Crozier and Stier, 1924–25, *b*). As an instance of the concordance among values of μ obtained from homologous activities, it may be added that this value also holds for the frequency of rhythmic gill contractions in larval *Amblystoma*. (The same magnitude holds after destruction of the forebrain of the salamander; we intend to describe these experiments separately.)

Destruction of the forebrain of the frog usually results in temporary acceleration of the breathing rhythm, followed by its very gradual decline. This was studied by means of frequent series of observations, over the temperature range, in a number of preparations, for periods

up to several weeks.   The method of observation is described in a
previous paper (Crozier and Stier, 1924–25, *b*).

It was earlier shown that the adjustment of frogs to laboratory
temperature involved the disappearance of a tendency to pronounced
decrease in frequency of pharyngeal movements, without change of
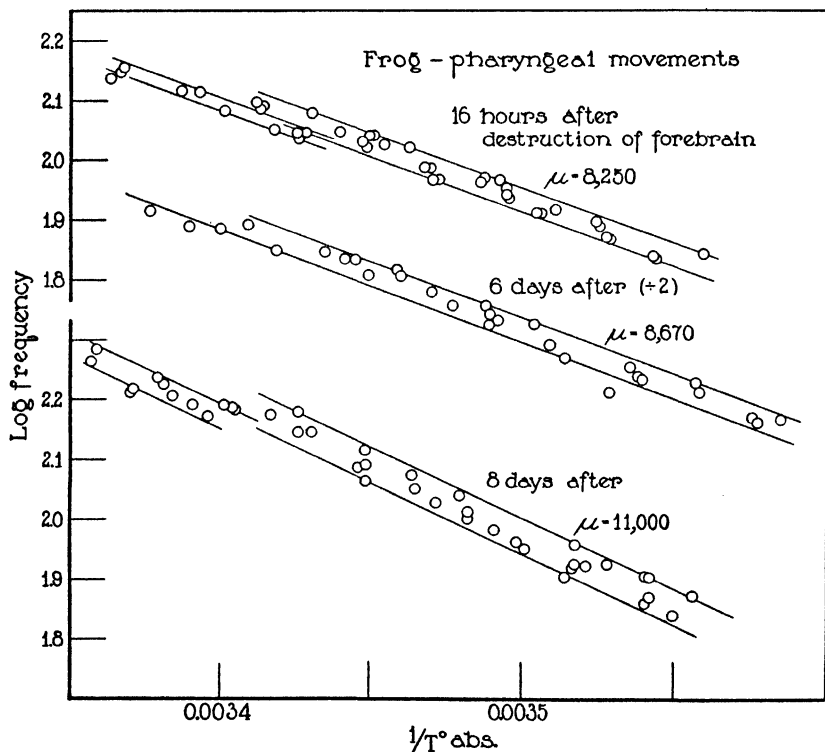
FIG. 3. Frequency of pharyngeal breathing movements in Frog 3, at intervals
after destruction of the forebrain.   The plotted points are averages of concordant
readings.

increment, at temperatures above 15°.   This result can be paralleled
by other temperature relations in frog tissues, and there seems no
doubt of its reality.   One effect of decerebration is to cause its more
or less abrupt reappearance.   For some days following decerebration
the best assignable temperature characteristic remains 8,500 ± 200
(Fig. 3).   The latitude of variation at given temperature is fairly

constant for each individual. About 8 days after destruction of the forebrain, in some cases, the increment is definitely altered. It now becomes $\mu = 11,000 \pm 50$, and the character of the break is changed (at least temporarily). The change appears later in other instances. At this time the frequency of pharyngeal breathing is detectably increased, at given temperature. Later the break already referred to may become very pronounced, without, in our observations, any
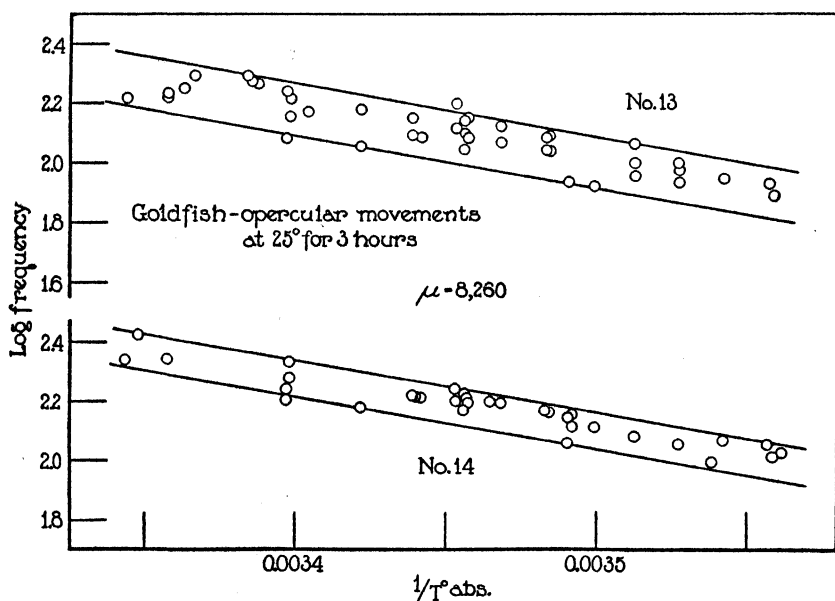
FIG. 4. Frequency of opercular movements in two goldfish following 3 hours exposure to a temperature of 25°. The plotted points are single observations. No. 13 showed constant gentle movements of the pectorals; No. 14 was quiet, with no fin movements.

further change of increment. We are not yet able to decide the exact mode of transition from $\mu = 8,500$ to $\mu = 11,000$, but we are confident that it does not involve a *gradual* upward tendency.

This sort of change of increment is not so convincing, by itself, as a similar one already described in grasshoppers (Crozier and Stier, 1924–25, a), where the sequence of changes in μ following decapitation is 7,900, 16,200, 11,200. We are able to add, however, one other instance among vertebrates in which the increment 8,000+ appears in

connection with an activity for which μ = 16,500 is characteristic. Two experiments with goldfish are given in Fig. 4. They show that it is possible to produce for opercular movements, instead of the increment 16,500, the value 8,300. This was accomplished by exposing the animals, previously ascertained to yield μ = 16,500 for frequency of opercular rhythm, to a temperature of 25° for 3 hours in water with oxygen content of about 3 cc. per liter. The increment 8,300 persisted for some hours after this treatment.

Thus in the breathing rhythm of vertebrates the increments 8,000+, 11,000+, 16,000+ seem definitely associated, in such a way as to make possible their experimental interconversion. The evidence from experiments with grasshoppers shows a similar system of processes to be controlling the frequency of breathing movements. It may be added that experiments with the rhythm of the book-gills of *Limulus* (Crozier and Federighi[3]) confirm this generalization so far as it relates to μ = 8,000+ and 16,000+. The respiratory movements of *Sepia*, according to some rather fragmentary data of Polimanti (1912), show μ = 11,000±.

III.

In commenting upon the temperature characteristics for heart beat frequency in molluscs, it was pointed out (Crozier and Stier, 1924–25, *d*) that this quantity differed in several good series of data for different species, but that the values of μ seemed to be members of an organically connected series frequently represented in vital processes and particularly associated with respiration (Crozier, 1924–25). Values of μ obtained by Glaser (1925–26, *b*) strengthen the basis for this hypothesis.

Attention was called to the fact that among pulmonates there seemed to be evidence of seasonal alteration in μ. This invited further attempts to modify, predictably, the magnitude of the temperature characteristic for heart rate in *Limax maximus*. Experiments during March, 1925 (in New Jersey), gave for *Limax* hearts μ = 16,300. A series of tests with animals freshly collected from a greenhouse late in December, 1925 (at Cambridge), gave uniformly

[3] Unpublished experiments on *Limulus*.

$\mu = 11{,}500$.   The technique was similar in both series of observations. The animals were each placed at the center of a rather snugly fitting long glass tube connected at one end to the outside air and at the other to a suction pump.   At intervals, between readings, the air in the tube was renewed with outside air (first brought to thermostat temperature).   By means of a lens of 8 inches focal length light from a pro-
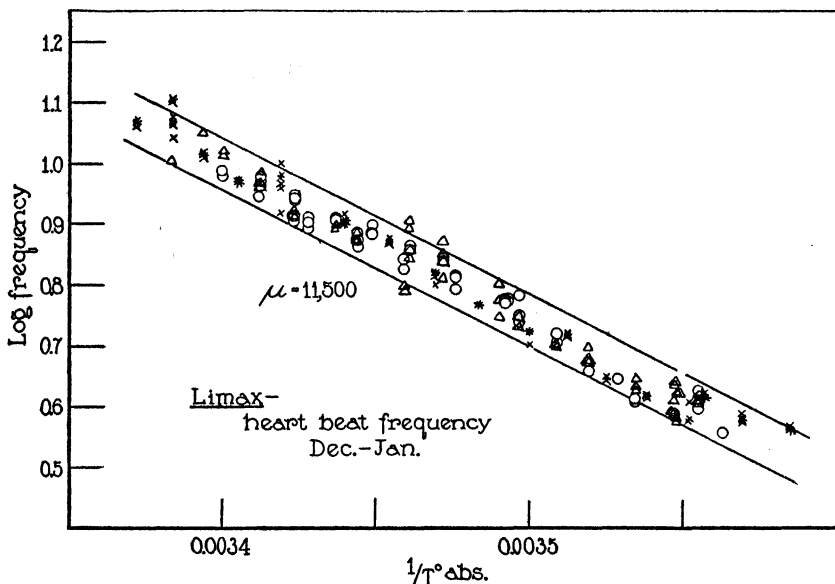
FIG. 5. Heart beat frequency as controlled by temperature, in *Limax* during December and January.   Three individuals.   The latitude of variation corresponds, at the highest temperature, to a variation of 1.35 sec., at the lowest temperature to 5.5 sec., in the time for 10 beats.   Above 25°, and below 9–10°, the animals are very difficult to keep quiet; with changes in peripheral tension the heart frequency rises.   The plotted points are single observations, from three animals (different symbols), brought together for comparison by the multiplication of the observations for two of the animals by a factor.

jection lantern, after passing through an infra-red filter, was concentrated upon the region of the heart of each *Limax*.   The tubes containing the animals were immersed in a water thermostat with parallel glass walls.   By this arrangement, the heart beat can be seen with little or no difficulty.   It is absolutely necessary that the

slug be motionless, with tentacles retracted, if any regularity is to be expected in the variation of the heart beat with temperature. Other things equal, the frequency of heart beat varies with size of the animal, smaller animals having a higher rate. Otherwise, no significant individual differences are found.

The two magnitudes, 16,300 and 11,500, agree quantitatively with those calculated for cardiac rates in the mussel *Anodonta* (11,200; Crozier and Stier, 1924–25, *d*, from Koch, 1916–17), the heteropod *Pterotrachea* (11,200 ± 500; Glaser, 1925–26, *b*), and in the pteropod *Tiedemannia* (16,200 ± 200; Glaser, 1925–26, *b*). There is evidence of their occurrence in *Helix* (data of Lang, 1910, in Crozier and Stier, 1924–25, *d*). They represent temperature characteristics or critical increments of processes commonly found associated with respiratory movements among arthropods (Crozier and Stier, 1924–25, *a*, grasshoppers; Crozier and Federighi[3]) and in the goldfish (Crozier and Stier, 1924–25, *c*), and which appear to be specifically implicated in respiratory metabolism and in phenomena assumed to be directly dependent thereon (Crozier, 1924–25, *a;* Glaser, 1924–25, *a;* Orr, 1924–25).

On the assumption that seasonal metabolic differences, connected with hibernation, may modify the temperature characteristic for heart rate in such pulmonates as *Helix* and *Limax*, it was sought to control this modification by the injection of sugar solution. The experiment is particularly interesting in the case of *Limax*, for after the ingestion of sugar there is independent evidence of a definite neuromuscular effect, namely the (reversible) suppression of phototropism (Crozier and Libby, 1924–25). But it is of course not to be presumed that the (central nervous ?) inhibition of phototropism connotes necessarily a central nervous effect which would inevitably be the one reflected in the (neurogenic ?) heart rhythm of *Limax*.

During the winter months *Limax* sufficiently large for these experiments are very difficult to obtain, due to hibernation, and only small numbers could be obtained from greenhouses. Those so obtained are distinctly less active at room temperature than is typical in spring. It is to be supposed that the nutritive level of such winter animals is distinctly lower than during warmer months. Certainly they eat less. Significant changes in reducing power associated with the state of nutrition are known to occur in the body fluids of other inverte-

brates (Morgulis, 1921; Hemmingsen, 1924), and presumably occur in *Limax*. We have ascertained that the Cu-reducing power of *Limax* blood is increased after ingestion of carbohydrate.

By moistening the lips of a slug with sugar solution, the mouth and pharynx are caused to open, so that the tip of a pipette may be
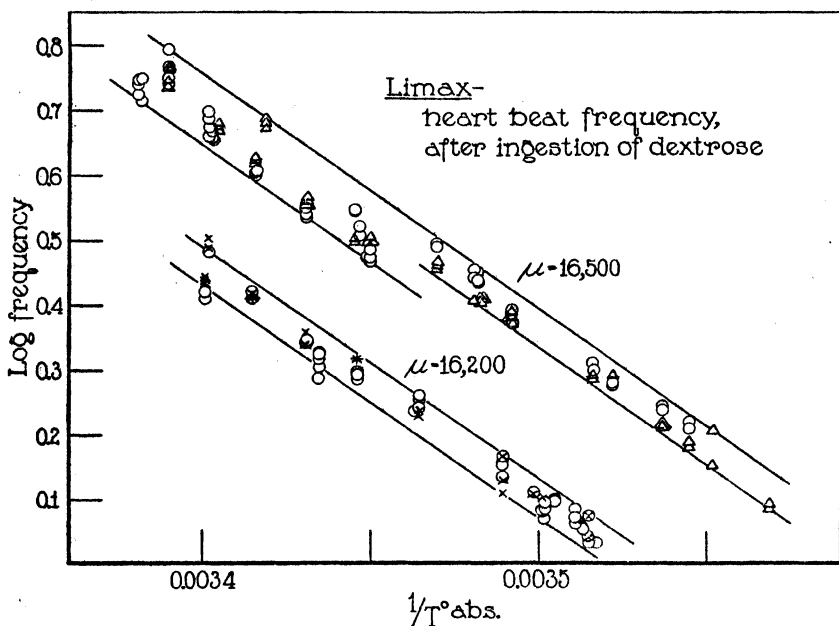
FIG. 6. Two sets of readings on each of two individuals, 6 to 24 hours after ingestion of 0.4 ± cc. M/5 dextrose. The development of an increased latitude of variation above 15°C. is more or less characteristic.

inserted for some distance. In this way 0.5 cc. of M/5 dextrose was placed within the alimentary tract. In the course of a few hours, during which the heart rate is very irregular, in correlation with muscular movements, the animals, which previously were negatively phototropic, become indifferent to light. Determinations of the temperature effect on the heart were begun about 6 hours after the ingestion of sugar. The effect of the sugar in connection with the heart apparently lasts for several days, but negative phototropism returns after about 48 hours.

The following account is based especially upon series of readings of heart beat frequency at different temperatures in two individuals most extensively used. The plotted points (Figs. 6, 7) are single observations, not averages. The total number of readings was 740.

Preliminary determinations (Fig. 5), four independent series on different days, gave $\mu = 11{,}500 \pm 250$. The last of these series was
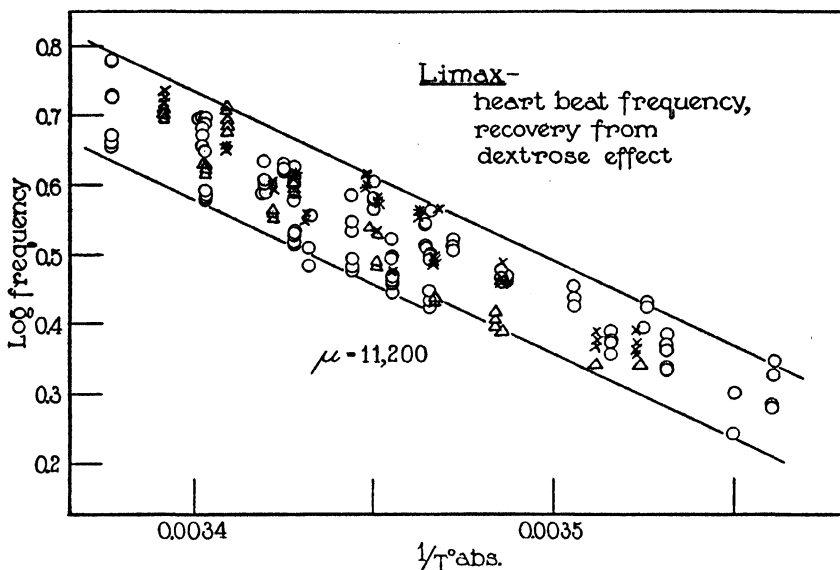
FIG. 7. Observations from the individuals involved in Figs. 5 and 6, showing recovery from temporary manifestation of $\mu = 16{,}200$ for heart beat frequency and the resumption of the value typical for the winter condition. The latitude of variation is greater than at the beginning of the experiment.

obtained on January 6, 12 days after the first. Thus there is a good indication of a definite, uniform value of the temperature characteristic under these conditions.

During 1 to 2 days after feeding the result of six series of observations, three on each animal, gave uniformly $\mu = 16{,}200 \pm 320$. This agrees quantitatively with the value previously obtained with active animals in late spring.

The probability is therefore great that one has in this case accomplished a definite alteration of the processes governing the frequency of the heart beat, of such a character as to experimentally

bring into a position of control one of two processes known to be associated in the matter. That the particular increment expected in this instance is in fact the one to appear is the best possible evidence that temperature characteristics correspond to discrete phenomena in living matter, which may in this way be classified and possibly identified as to their chemical nature.

IV.

The question at once arises: Is the effect of the sugar ingestion reversible? There are two possibilities. Either the animal is permanently aroused from its semiestivation, or the effect on the heart beat is due merely to a temporary metabolic disturbance. In the latter event the demonstration of experimental control of the temperature characteristic is just so much neater. But the former possibility would not affect the interest of the change from one specific value to the other.

The increment 16,300 consequent upon sugar ingestion does in fact disappear, and within at least 4 days the temperature effect on the hearts in these experiments reverts to the initial value, 11,000+. The actual magnitude obtained 6 days subsequent to sugar ingestion is 11,200 ± 300, in four series of measurements (see Fig. 7).

V.

SUMMARY.

In December and January the frequency of heart beat in *Limax* exhibits $\mu = 11,500 \pm 250$. The ingestion of a small volume of sugar solution results in temporary change of μ to 16,200 ± 320, which accords quantitatively with the value obtained from these slugs in spring. This effect of the sugar is reversible, but lasts longer than the abolition of negative phototropism which the sugar also produces.

Other instances are given in which the value of the temperature characteristics for vital processes have been changed experimentally. The new values which appear have already been obtained in connection with homologous activities.

These results confirm the view that the critical thermal increments serve to characterize recognizably different governing reactions in living matter, and indicate a basis for specific experimental control.

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MECHANISM OF THE ACCUMULATION OF DYE IN NITELLA ON THE BASIS OF THE ENTRANCE OF THE DYE AS UNDISSOCIATED MOLECULES.

By MARIAN IRWIN.

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I.

INTRODUCTION.

The mechanism of the accumulation of the basic dye, brilliant cresyl blue, in the sap of *Nitella* has been discussed by the writer[1] on the basis of experiments made with different concentrations of the dye at one pH value. More recently a preliminary report[2] was made by the writer on experiments[3] with one concentration of the dye at different pH values. A fuller account of these experiments is given in the present paper. In order to understand the mechanism it is necessary to analyze the data for (1) the rate of penetration of the dye into the cell sap. and for (2) the final equilibrium.

II.

Methods.

The living cells[4] of *Nitella* were placed at 25°C. $\pm$ 0.5° in 2×10^{-6} M dye solutions (brilliant cresyl blue) at different pH values, from pH

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[1] Irwin, M., *J. Gen. Physiol.*, 1925–26, viii, 147.

[2] Irwin, M., *Proc. Soc. Exp. Biol. and Med.*, 1925–26, xxiii, 251.

[3] Experiments such as these were made by the writer on *Nitella* found in Woods Hole, Massachusetts, with brilliant cresyl blue made by the National Aniline Chemical Company, but at that time the dissociation constant of the dye was not known, so that the analysis of the data was not complete. Since it is no longer possible to obtain the *Nitella* at Woods Hole, the experiments were repeated with *Nitella* found at Cambridge, and with the dye made by Grübler, the dissociation constant of which was found by the writer as will be described later. Irwin, M., *J. Gen. Physiol.*, 1922–23, v, 727.

[4] For details of technique see the writer's paper referred to in Foot-note 1. The *Nitella* used was obtained from Cambridge, Massachusetts.

6.1 to pH 9.3 (M/150 phosphates or borates as buffers).[5]   The concentration of the dye was kept constant throughout each experiment. At definite intervals the cells were removed from the solutions.   The end of each cell was then cut and the sap was squeezed out upon a glass slide.   The sap was then drawn up into a capillary tube, the color of which was matched with that of the capillary tube containing the standard dye solution.

<p style="text-align:center">III.</p>

Analysis of the Rate of Penetration.

When the concentrations of the dye in the sap were thus determined at definite intervals, it was found that the greater the pH value of the external solution, the higher was the rate of penetration.   The maximum was reached at about pH 9.3, where further increase in pH value of the external solution brought about no appreciable increase in the rate of accumulation.   We may assume that the dye behaves as a weak base and that the dye ions cannot enter but that the dye penetrates only in the form of undissociated molecules of the free base which for convenience will be referred to as DOH.   In that case, the rate of penetration at the start should be proportional to the concentration of DOH in the external solution.   With a constant concentration of the dye the concentration of DOH will depend on the pH value.   If this assumption is correct, we can calculate the concentration of DOH, expressed as per cent of the total dye present from the rates of penetration into *Nitella*, and this should agree with the values obtained by other methods such as that of determining the distribution[6] of

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[5] The readings made at pH 6.1 are rather uncertain because at this pH value the cellulose wall becomes deeply stained, and it is difficult to avoid contamination of the sap from the dye adhering to the cell wall at the cut end.   The readings made with pH values lower than this cannot be used since the lower the pH value the more rapid is the staining of the cellulose wall and the greater the chance of contamination.

[6] Pure chloroform was added to 1.4×10^{-5} M dye solution previously saturated with chloroform at different pH values (M/150 phosphates or borates) at 24°C. $\pm$ 1°, and the determination of the amount of dye taken up by chloroform at equilibrium was made colorimetrically.   The color of the dye in water is blue at the pH values used, but in chloroform it is pink (when the dye comes out again into water it is blue).   In order to make the colorimetric determinations accurate,

the dye between chloroform and water, which was employed by
the writer.

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it was necessary to use different volumes of chloroform and the dye solu-
tions at different pH values, so that at equilibrium the concentration of the
dye will be reduced to about 0.000007 M. Such mixtures were shaken vigorously in
a separatory funnel, and after equilibrium was established the chloroform was al-
lowed to separate and was then drawn off. The aqueous solution was then col-
lected in a test-tube, and tightly stoppered at once. Extreme care must be taken
to avoid the slightest evaporation of the chloroform in the funnel or in the test-
tube, or else the aqueous solution will at once become more concentrated by taking
up the dye left by the evaporated chloroform. The color of the tube containing
the aqueous solution was matched with that of tubes of the same diameter con-
taining standard dye solutions. Since the volume of the aqueous solution, the
volume of the chloroform, and the concentration of the dye in the aqueous solution
at start and at equilibrium were known, the concentration of dye in the chloroform
at equilibrium could be readily calculated. When this was done, it was found that
the relative amount (distribution coefficient) of dye taken up by the chloroform
increased with increase in the pH value of the aqueous solution, until a maximum
was reached at about pH 9.3, when further increase in the pH value brought about
no appreciable increase in the taking up of the dye. This was not due to the
saturation of the dye in the chloroform, because more dye was taken up on raising
the concentration of the dye in the aqueous solution. At this pH value it may be
assumed that 88 per cent of the dye in the aqueous solution is in the form of un-
dissociated DOH. On this basis, it is possible to calculate the value of the constant
at the pH value where 100 per cent of the dye is undissociated DOH, by the equa-
tion $\dfrac{C_1}{C_2 (1 - \alpha)} = K$, in which C_1 is the concentration of undissociated DOH in
chloroform, C_2 is the concentration of the total dye in water, K is the constant,
and α is the molar fraction of the dye dissociated. By substituting 0.88 for α
and the observed values of C_1 and C_2 at pH 9.3, the value of K was determined
where all of the dye in the aqueous solution was in the form of undissociated
DOH, and was found to be 780.

There seems to be no association of the dye in the chloroform because dilution
of the aqueous dye solution does not change the value of the constant.

The degree of dissociation of the dye may now be calculated at different pH
values by using the above equation.

The percentage of undissociated DOH calculated in this manner for various pH
values of the aqueous solution from pH 5.3 to 9.3 is shown by the symbol $\times$ in Fig. 1.
From this curve the dissociation constant of DOH is determined graphically
to be $10^{-5.6}$. Cf. Michaelis, L., Die Wasserstoffionenkonzentration, Berlin, 2nd
edition, 1922, 44, 46, in which pOH is substituted for pH and OH for H.

This method was checked by another in which the chloroform containing the
dye was removed from the aqueous solution and placed in 2 cc. of water after

When the rates[7] (the reciprocal of time taken for the concentration of the dye in the sap to reach 3.45×10^{-5} M) for different pH values of the external dye solutions are calculated, it is found that a maximum is reached at about pH 9.3, as already stated. This is regarded as indicating that the percentage of undissociated DOH has nearly reached its maximum value. We assume[8] that this is 88 per cent of the total dye and the per cent of undissociated DOH at different pH values is calculated on this basis by assuming that the rate of penetration is directly proportional to the concentration of DOH. When such values are plotted against the external pH values, the curve agrees closely with that obtained by the experiments on the distribution of the dye between chloroform and water, as shown by the symbols $\times$ and $\bigcirc$ in Fig. 1. The theoretical curve, calculated from the dissociation constant of the dye,[9] $K = 10^{-5.6}$ follows these two curves,

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which the chloroform was driven off by a current of air. After complete evaporation of the chloroform the solution was diluted to a point at which a good colorimetric determination could be made. The results thus obtained agreed closely with those described above.

Distribution of the dye between benzene and water was determined at different pH values. The constant, K, of the partition coefficient was found to be lower than for chloroform so that for very high pH values it was more satisfactory but for pH values below 8 it was so unsatisfactory that the results obtained by this method were not seriously considered. The dissociation constant was found to be about $10^{-5.2}$.

[7] The rates taken with 0.000014 M dye in the sap gave the same type of curve. The rates were taken at a low concentration to avoid the possibility of error from having the pH values of the sap affected by the dye. The results seem to indicate that so long as the concentration of the dye in the sap does not go above 0.0000345 M such errors as the above described are avoided. If we compare the amounts of dye taken up by the sap at different pH values near the start of the experiment (at 3 minutes) we get the same type of curve.

[8] This value was chosen as producing the best agreement among the curves shown in Fig. 1.

[9] The equation used for the calculation of α, the fraction of the dye dissociated is:

$$\alpha = \frac{1}{1 + \dfrac{OH}{K}}$$

in which K is $10^{-5.6}$. In a previous paper (Foot-note 1) this was stated as K = $10^{-6.4}$. Since, however, the dissociation constant could not be accurately determined at that time, the calculations were not published in detail. This has been remedied by improved technique.

as shown by the curve as drawn in Fig. 1 until about pH 7, below which the chloroform curve becomes lower than the calculated (unfortunately this difference is not well marked in the figure because the scale
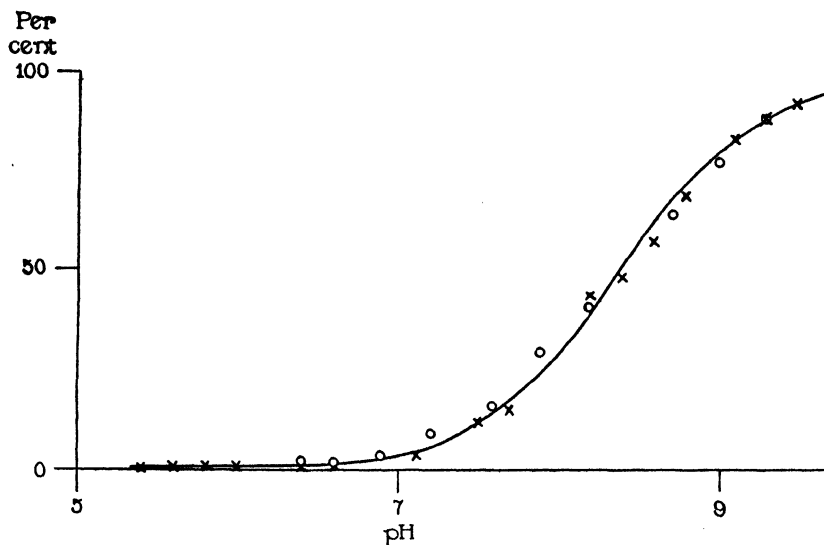
Fig. 1. Curve showing the percentage of undissociated molecules of brilliant cresyl blue at different pH values. The ordinates represent the percentage and the abscissæ represent the pH values. Symbol $\bigcirc$ represents the data from rates of penetration of the dye into *Nitella*. Symbol $\times$ represents the data from the distribution of the dye between chloroform and water. The curve as drawn represents the calculation made from the equation, $\alpha = \dfrac{1}{1 + \dfrac{OH}{K}}$, when $K = 10^{-5.6}$.

for plotting is very small at these pH values). This difference may be due either to experimental errors or to the dissociation of a second salt-forming group in the dye molecule. If we put $K_1 = 10^{-5.6}$ and $K_2 = 10^{-8}$ the theoretical[10] curve agrees more closely with the chloro-

[10] The equation used for the calculation of ρ, the fraction of the dye undissociated is $\rho = \dfrac{1}{1 + \dfrac{K_1}{OH} + \dfrac{K_1 K_2}{(OH)^2}}$ in which $K_1 = 10^{-5.6}$, and $K_2 = 10^{-8}$.

form curve, but since the chances for experimental errors are rather great at these lower pH values, the writer does not wish to make a definite statement as to the nature of this difference until an opportunity presents itself to determine the dissociation constants more accurately.

These results seem to indicate that the dye enters only in the form of DOH, and that the rate of penetration is directly proportional to the concentration of DOH in the external solution, provided the conditions in the cell are kept constant. Changes in the condition of the cell sap, for example, can alter the rates of penetration, as has already been shown,[11] though none of the experiments are very reliable since the cells might have been injured with the changes in the pH values of the sap. The sap of *Nitella* is buffered, according to Hoagland and Davis,[12] so that, in all probability, the presence of 3.45×10^{-5} M dye in the sap brings about no change in the pH value of the sap. Even if the pH values are increased by the presence of this amount of dye in the sap, the relative rates will not change so long as the pH value of the sap is changed to the same extent for all external pH values.

[11] McCutcheon and Lucke, and later the writer, have found that an increase in the pH value of the sap brought about a decrease in the rate of penetration (cells may be injured). Recently the writer has found that when acetic acid penetrates the living cells of *Nitella* until the pH of the sap is changed from pH 5.6 to 5, an increase in the rate of penetration of the dye takes place. This experiment is unreliable because there is formed a white precipitate in the sap (in all probability the protein in the sap has reached its isoelectric point), and the cells may be injured. The fact that after a brief exposure to NH₃ the rate may be decreased before the pH value of the sap is increased may be due to the fact that the NH₃ is at that time confined to the protoplasm and the outer portion of the sap, where it could affect the rate by locally raising the pH value without, however, affecting the pH value of the sap as a whole when squeezed out on the slide, or due to the fact that in correspondence with the conditions in the sap, there is present NH₃ (without the change in the pH value) in the parts of the cell other than the vacuole (McCutcheon, M., and Lucke, B., *J. Gen. Physiol.*, 1923–24, vi, 501. Irwin, M., *J. Gen. Physiol.*, 1925–26, ix, 235.)

[12] Hoagland, D. R., and Davis, A. R., *J. Gen. Physiol.*, 1922–23, v, 629.

IV.

Analysis of the Equilibrium.

The experiments described in Section II may be regarded as indicating that the dye enters the cell only in the form of DOH. It is desirable to inquire whether the analysis of the equilibria will support this interpretation.

At pH 6.4, 6.6, and 6.9, the absorption of dye reaches an equilibrium, but at higher pH values of the external dye solutions the cells die before the equilibrium was attained. The equilibrium values thus obtained increase as the pH values of the external solutions rise.

If the dye penetrates as DOH we shall expect that at equilibrium the internal and external concentrations of DOH will be the same. Thus when the external dye solution is 2×10^{-5} M and the external pH is 6.9, the concentration of DOH in the external solution may be taken as 3.16 per cent of 0.00002 M (since according to the theoretical curve, 3.16 per cent of the dye is in the form of DOH at pH 6.9). Hence we have 6.31×10^{-7} M DOH in the external solution and in the sap at equilibrium. In the sap the pH value may be taken as 5.6, at which value DOH forms 0.16 per cent of the total dye (according to the theoretical curve). Hence when DOH enters the sap it must dissociate, forming a sufficient number of ions to constitute 99.84 per cent of the total dye inside. If we assume that these ions cannot escape from the cell vacuole, then the total dye, x, inside will be

$$x = \frac{100}{0.16} \times 0.000000631 \text{ M} = 0.000395 \text{ M}$$

whereas we actually find 0.00014 M by observation.[13] Table I shows the corresponding values calculated for the pH values 6.4 and 6.6.

At all of the pH values the calculated values are higher than the observed.

If the above assumption is correct, then it should be possible to treat in the same manner the previous determinations[1] of the concentrations of the dye in the sap at equilibrium with different concentrations of external dye solutions at one pH value. When such

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[13] *Cf.* Osterhout, W. J. V., and Dorcas, M. J., *J. Gen. Physiol.*, 1925–26, ix, 255.

TABLE I.

From the percentage dissociation of brilliant cresyl blue calculated when $K = 10^{-5.6}$ the values of the total dye (D^+ ions and DOH) in the sap of *Nitella* at equilibrium are calculated at different pH values of the external dye solutions at 25°C. The concentration of the external dye solution is 2 M $\times 10^{-5}$, and the pH value of the sap is 5.6, at which pH value 0.16 per cent of the total concentration of the dye is undissociated. The calculations for Tables I and II were made with a 20 inch slide rule.

pH of the external dye solution.	Concentration of undissociated molecules in the external solution.	Concentration of undissociated molecules in the sap and in the external solution.	Observed values of the total concentration of the dye in the sap.	Calculated values of the total concentration of the dye in the sap.
	per cent	M $\times 10^{-5}$	M $\times 10^{-5}$	M $\times 10^{-5}$
6.4	1	0.03	6	12.5
6.6	1.6	0.036	9	20.0
6.9	3.16	0.076	14	39.5

TABLE II.

From the percentage dissociation of brilliant cresyl blue calculated when $K = 10^{-5.6}$ the values of the total dye (D^+ ions and DOH) in tne sap of *Nitella* are calculated for different concentrations of external dye solutions at 25°C. The pH of the sap is 5.6, at which pH 0.16 per cent of the total concentration of the dye is undissociated. At the pH value of the external dye solution (pH 6.9) 3.16 per cent of the dye is undissociated.

Concentration of external dye solution.	Observed values of total concentration of dye in the sap.	Calculated values of the total concentration of dye in the sap.
M $\times 10^{-5}$	M $\times 10^{-5}$	M $\times 10^{-5}$
0.40	2.8	7.9
0.65	4.4	12.8
1.00	6.9	19.8
1.30	9.0	25.7
1.5	11.0	29.6
1.7	12.4	33.5
2.0	14.1	39.5
2.6	27.5	51.3
3.1	32.0	61.2
4.1	46.5	81.0

calculations are made, it is found, as shown in Table II, that the values of the total dye in the sap are higher than the observed. The fact that the observed values are lower than the calculated cannot be

wholly due to the increase in the pH value of the sap brought about by the presence of the dye in the sap, because, if this were the case, the extent of the lowering of the concentration of the dye in the sap should increase proportionally as the concentration of the dye in the sap is increased, but this does not seem to be the case. Such a lowering may be due to the fact that the dye is not so soluble in the sap as it is in the external solution or that the dissociation constant of the dye is not the same in the sap as it is in the external solution. Unfortunately there is not sufficient quantity of sap available to determine this point.

When the concentration of the dye has reached about 0.00014 M in the sap, the disagreement between the observed values and the calculated becomes less. This may be due to the occurrence of secondary changes in the cell, which increase the final concentration of the dye in the sap, as already suggested[1] by the writer.

If the values of the dye in the sap are calculated on the assumption that there are two dissociation constants, by using the values for the undissociated DOH calculated from the equation[10] already described, the discrepancy between the calculated values and the observed values of the dye in the sap is still greater.

Furthermore, if a correction is made for the ionic strength of the sap (about 0.1 M, comprising NaCl and KCl in about equal proportions) the discrepancy becomes still greater.

Let us now see if the values calculated on the basis of the Donnan equilibrium which is based on the entrance of ions will not agree with the observed. According to the Donnan equilibrium the relation $\dfrac{H^+ \text{ inside}}{H^+ \text{ outside}} = \dfrac{D^+ \text{ inside}}{D^+ \text{ outside}}$ must hold if the dye behaves as a monoacid base. When the values of D^+ ions inside are calculated on this basis, it is found that the calculated values are higher than the observed to the same extent as found in the case of the values calculated on the basis of the entrance of the dye as undissociated molecules of DOH when $K = 10^{-5.6}$

If the dye behaves as a diacid base with K_1 equal to K_2, the relation $\dfrac{D^+ \text{ inside}}{D^+ \text{ outside}} = \dfrac{(H^+)^2 \text{ inside}}{(H^+)^2 \text{ outside}}$, in which case the discrepancy between the observed and the calculated values is still greater.

In case K_1 is not equal to K_2 the calculation of the dye in the sap is somewhat complicated.

Whether the dye behaves as a monoacid or diacid base it would not be possible to distinguish from an analysis of the conditions in the sap at equilibrium if the dye enters the cell as undissociated dye base or as ions. We cannot assume that $\dfrac{H^+ \text{ inside}}{H^+ \text{ outside}} = \dfrac{D^+ \text{ inside}}{D^+ \text{ outside}}$ unless H^+ ions are diffusible through the protoplasm, but such is not the case with *Nitella*. The Donnan equilibrium requires that all the diffusible cations should stand in the same relation (inside to outside) as the H ions, but this is not the case. Furthermore the relation of Cl ions should be the reverse of that of the cations, but as a matter of fact the contrary is true. It therefore does not seem probable that the results can be explained on the basis of the Donnan equilibrium. Moreover, in all probability the ions do not enter, as pointed out by Osterhout and Dorcas,[13] because, if the rate of penetration increases with increase in the outside concentration of undissociated molecules the conditions at equilibrium cannot be due to the Donnan effect unless the undissociated molecules penetrate much more rapidly than the ions.

<div align="center">

V.

DISCUSSION.

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The above analysis seems to indicate that the rate of penetration of the dye into living cells of *Nitella* is proportional to the concentration[14] of undissociated molecules of the dye in the external solution, provided the conditions in the cell remain the same for all the external pH values. Since the temperature coefficient between 20° and 25°C.

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[14] The following writers have assumed that a basic dye enters a living cell in the form of undissociated molecules, Overton, E., *Jahrb. wissensch. Bot.*, 1900, xliii, 669. Harvey, E. N., *J. Exp. Zool.*, 1911, x, 507. Robertson, T. B., *J. Biol. Chem.*, 1908, iv, 1. McCutcheon, M., and Lucke, B., *J. Gen. Physiol.*, 1923–24, vi, 501. Brooks, M. M., *Proc. Soc. Exp. Biol. and Med.*, 1925–26, xxiii, 265. Referring to her experiments on the penetration of 2, 6-dibromophenol-indophenol into *Valonia*, Brooks states that "the amount of dye in the sap at equilibrium is proportional to the amount of undissociated dye in the external solution." Without further details, the writer is unable to determine whether in this case the dye enters as undissociated molecules or not.

is about 4.8, the rate cannot be dependent on the simple diffusion of DOH into the cell vacuole. The process may be complicated by a chemical combination of the dye in the protoplasm, or in the membranes, which might be a slower process than diffusion. This idea is supported by the following observation. In the experiments of Osterhout and Dorcas[13] on the penetration of CO_2 into living cells of *Valonia*, the temperature coefficient is very low (that of diffusion), while in the case of the writer's experiments on the penetration of brilliant cresyl blue into *Valonia* the temperature coefficient is very high (that of a chemical reaction). This leads the writer to believe that CO_2 enters the cell vacuole without combining with protoplasmic constituents, while the dye enters into combination. It may be possible that the dye enters by diffusion complicated by some other factors which are unknown to us at present.

In either case, it might well happen that the time curve of penetration of the dye into the cell vacuole would follow the equation for a unimolecular reaction as described.[1]

The treatment of the time curves made in the writer's previous papers on the penetration of the dye into the living cells of *Nitella* will hold on the basis of the present theory.

As to the conditions at equilibrium, the analysis seems to indicate that the final concentration of the dye is governed by the concentration of DOH in the external solution and by the percentage of dissociation of DOH in the sap (provided there are no complications due to other factors), as previously suggested by Osterhout and Dorcas[13] in discussing the penetration of CO_2 into *Valonia*.

In case there is a combination of the dye with a constituent, XA, of the sap, according to the equation $DOH + XA \rightleftharpoons DA + XOH$, the total dye in the sap would be composed of DOH, D^+ ions, and DA (all of the same color), and the calculations would have to be made accordingly. If DA were slightly soluble or slightly ionized, the concentration of D^+ ions and of DOH would remain the same as if DA were not present, unless the solubility or the pH values are changed by the presence of DA.

It may be added that all that has been said regarding DOH would apply equally well to a tautomer of the dye which acts similarly to DOH.

The solubility of the dye in the sap is also an important factor to be considered in relation to the penetration.   It is of interest to mention here that methyl red, even at pH 8 or 9, where the dye is practically in the form of undissociated DOH and can be readily absorbed by the chloroform, cannot enter the cell sap, and it may be that this is due to the fact that methyl red is not very soluble in the sap.   Or, it may be possible that there is a specificity in the behavior of the cell toward the undissociated molecules.   That not all undissociated molecules enter may be still further shown by the fact that the acid dyes, such as thymol blue, brom thymol blue, phenol red, brom cresol purple, at pH 5.5, where the greater percentage of the total dye is in the form of undissociated HD molecules, do not enter the cell.   These indicators are not very soluble in aqueous solution and in chloroform, so that this may be interpreted as being due to the still greater lack of solubility of the dye in the sap and in a lipoid, but a dye, such as acid fuchsin, which is readily soluble in water and slightly soluble in chloroform does not enter the cell.   Further investigation is now being undertaken, and in the near future the writer hopes to throw some light on this problem.

The assumption that the ions do not enter appreciably is still further supported by the experiments on other basic dyes, crystal violet, malachite green (nitrate), and tetramethyl diaminophenoxazonium nitrate, neutral red, and methylene blue,[15] the rate of penetration of which depends chiefly on the concentration of undissociated DOH molecules.

[15] The writer is indebted to Dr. W. A. Jacobs and Dr. M. Heidelberger of this Institute for their kindness in supplying her with the first three dyes in highly purified form.

The methylene blue was purified by repeated recrystallization and extraction with chloroform.   This dye does not appreciably enter the living cells of *Nitella* and *Valonia* at pH 5.4.   In case the ions enter they enter so slowly that it is difficult to determine whether or not the presence of the dye in the sap is due to the contamination of the sap from the stained cell wall, or to an injury. This result is contrary to the results obtained by Brooks[14] on the penetration of the dye into *Valonia*.   Methylene blue is not a good dye to use for this purpose because it is very difficult to separate it in pure form from other dyes which behave as weaker bases and which are mixed with it in great quantity, so that we cannot tell at higher pH values whether the dye which enters is methylene blue or other dyes which are not so strongly dissociated.

The mechanism of the penetration of the dye into living cells of *Nitella* represents by no means a simple process, and though the results tend to confirm more and more the assumption discussed in this paper, the writer disclaims any intention of attempting a complete explanation at present.

SUMMARY.

The rate of penetration of brilliant cresyl blue into the living cells of *Nitella* indicates that the dye enters only in the form of the undissociated molecule. At equilibrium the total concentration of the dye in the sap is proportional to the concentration of the free base in the outside solution.

THE EFFECTS OF CERTAIN HEAVY METALS ON RESPIRATION.

By S. F. COOK.

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I.

The experiments here described were performed to determine the effects of copper, silver, and mercury on respiration. For purposes of comparison it was found advisable to include hydrogen (as hydrochloric acid). The organism used was *Aspergillus niger*, a fungus which, because of the rapidity of its growth and the ease with which it is manipulated, lends itself readily to such investigation. The effect of the above mentioned four elements is to check the respiration of *Aspergillus* in a definite and characteristic manner. An exceptional result was found with copper, in the form of a latent period which lasted a considerable length of time before the toxic effect became evident, and which will be discussed in detail in another paper (see Fig. 1). In this paper the general toxic effects of copper, mercury, silver, and hydrogen are described, together with the special effects of temperature and concentration; and an hypothesis is advanced by means of which the toxic effects may be placed on a mathematical and predictable basis.

II.

Respiration was measured by the indicator method, with an Osterhout respiration machine, the principles of which have been often described (*cf.* articles by Osterhout, Haas, Inman, Ray, Gustafson, *et al.* in *The Journal of General Physiology*). There is a closed system of tubes containing the organism and a tube with a solution of the indicator phenolsulphonphthalein. The pumping system, by means of a bulb, sends a continuous current of air through the apparatus, carrying the carbon dioxide from the respiring organism to the indicator, which changes from light pink to yellow. The limits of the color change are fixed by two tubes of buffer solutions made up as known pH standards, in this case pH 7.36

and 7.09. When the indicator solution has been changed to match the lower standard the current is switched into a U-tube containing sodium hydroxide which removes the carbon dioxide. The air free from carbon dioxide is then run through the indicator which sweeps out what carbon dioxide is held in it, thereby raising the pH and restoring the color to that of the upper standard. The shifting of the direction of the current was done with a three-way stop-cock.

The organism was placed in a test-tube 1½ inches in diameter, fitted with a rubber stopper. When a solution was to be introduced after the beginning of the experiment it was done by means of a separatory funnel fused to the intake tube. The intake tube ran through the stopper to the bottom of the test-tube
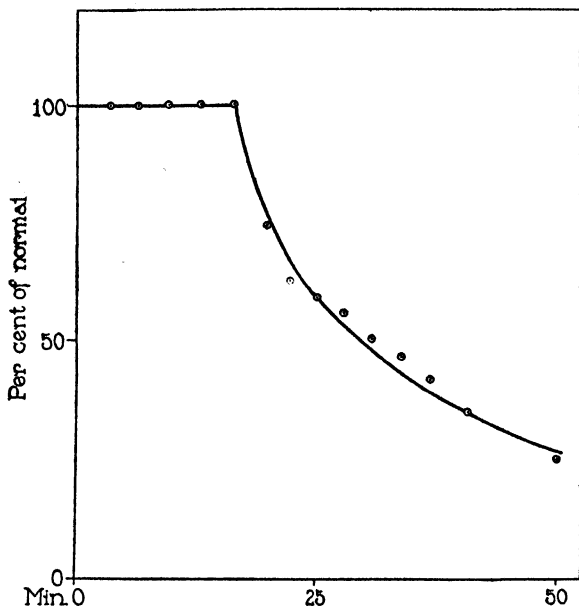
FIG. 1. Curve with copper chloride 0.25 M; K_v (monomolecular) = 0.022.

serving as respiratory chamber. In this way the liquid could be run directly into the chamber without opening the apparatus or allowing any outside air to get in. In some experiments it was necessary to replace the liquid in the chamber by an entirely fresh solution. For this purpose a tube was led off the closed system near the outlet from the chamber and connected with a U-tube filled with sodium hydroxide. A rubber tube was connected with the far side of the U-tube and a stop-cock closed the outlet securely when not in use. In this way carbon dioxide could be removed from air passing in or out of the apparatus. Another tube was run from the bottom of the respiratory chamber through the stopper to the outside and was likewise closed with a pinch-cock when not in use.

This system involved no significant leakage from the outside, as was determined by frequent tests. When it was desired to change the solution in the chamber, both exit tubes were opened and the liquid present was blown out, all carbon dioxide being removed from the incoming air by the sodium hydroxide in the U-tube. Then the pinch-cocks were closed and the new solution run in, in the usual manner, through the separatory funnel. This process was repeated as often as was necessary, in a very short time, and the organism could be washed quite free from the original fluid.

It was usually necessary to maintain a constant temperature. A thermometer was sealed into the respiratory chamber through the stopper. The whole chamber was set in a water bath with a mercury thermoregulator. The temperature was read from the thermometer inside and could be regulated to about 0.5°C., which was sufficiently close.

The normal respiration rate was obtained by determining the length of time to change the indicator from pH 7.36 to 7.09. When the toxic agent is introduced there is a change in the time of decolorization and hence a change in the rate of respiration. The indicator solution was made slightly alkaline (0.0001 M) with sodium bicarbonate. Ordinary tap water has been used, but only because there was about the right amount of alkali in it. Since the nature and quantity of this is unknown it is preferable to use distilled water and a known amount of the bicarbonate.

The results are expressed with reference to the rate of the carbon dioxide production and not with reference to absolute amounts. It is immaterial how much carbon dioxide is normally produced by the organism (within limits of convenience), because all subsequent changes are expressed as per cent of the normal. Thus it makes no difference if the total quantity of the fungus varies from experiment to experiment. The rate curves will always be comparable.

The organism, *Aspergillus niger*, was taken from a culture kindly supplied by Dr. W. H. Weston and has been kept in subcultures on potato agar. For use in these experiments it was grown on a liquid medium in Erlenmeyer flasks. The medium, adapted from that used by Gustafson in this laboratory (1918–19) was made up as follows: KNO_3, 2 gm.; KH_2PO_4, 0.75 gm.; K_2HPO_4, 0.25 gm.; $MgSO_4$, 0.5 gm.; sucrose, 40 gm.; and water 1000 cc. After being sterilized, the flasks were inoculated with a platinum or nichrome needle, special precautions being taken to avoid bacterial or fungus contamination. The mould was then placed in an incubator at about 30° and used after 48 to 60 hours. The growth was uniformly a solid mat which could be removed and handled easily. Pieces of appropriate size were cut out and washed free of the nutrient solution in distilled water. They were then placed in the respiration chamber in a 1 to 2 per cent glucose solution. The glucose was perhaps unnecessary, and no difference was ever detected in the toxic effect when the glucose was not used; but it seemed advisable not to force the mould to undergo so great a change in osmotic pressure as from the nutrient fluid to distilled water.

Aspergillus is a very resistant fungus and will tolerate extreme conditions, but

the respiration curves were found to be affected by two principal factors: (1) The rate of normal respiration varies with the temperature, as was to be expected. It was found that the rate of respiration increased with temperature to a maximum at about 40°C. and then fell off rapidly and ceased at about 50°C. It was impossible, therefore, to use temperatures above 40°C. (2) The fungus changes in its resistance to toxic solutions with age. The culture should be at approximately the same age throughout a series of experiments. The best age is from 2 to 3 days, although a little older material will give good results. Over 4 days it should not be used.

III.

When a solution of copper chloride is run into the respiration chamber containing the organism there is no change in the rate of respiration for a definite length of time which depends on the temperature and on the concentration of $CuCl_2$. Then suddenly the rate falls off and the carbon dioxide production gradually ceases (see Fig. 1). The drop is similar to that produced by many other substances, but the preliminary period during which there is no change is peculiar and has been found only with copper, iron, and tin. The interval observed with the latter two elements is much shorter than with copper. There are two names which might be applied to it: latent period or induction period. Of these the former seems more appropriate. The latter is a chemical rather than a biological term and is often taken to mean a period of slight activity followed by one of greater activity (Mellor, 1904), while here there is no detectable activity at all previous to the sudden change. This entire lack of activity is better described by the term latent period. Since the period occurs only with the three elements mentioned and has peculiar characteristics it will be discussed separately, and we will consider here only the drop in rate of respiration disregarding the latent period.

On analyzing the drop following the latent period it is found to follow approximately the course of a monomolecular reaction curve. In a monomolecular reaction there is a single substance undergoing decomposition and the amount decomposed in unit time is proportional to the amount present. Thus if a is the amount originally present, and x is the amount present after time t, then the rate of transformation $\frac{dx}{dt}$ is $K(a - x)$, or in the integral form, $K = \frac{1}{t} \log$

$\left(\dfrac{a}{a-x}\right)$. K is the velocity constant of the reaction. In the case of decreasing rate of respiration it might be termed the velocity constant of the toxic action.

In applying this formula to the curves obtained with copper and the other heavy metals it becomes evident that there is always a tendency for the value of K to diminish as greater and greater values
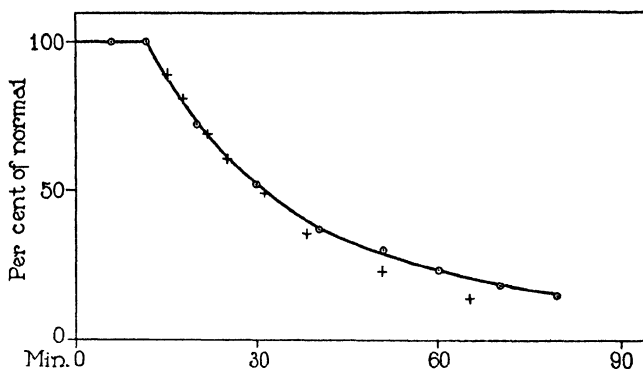
Fig. 2. Curve with copper chloride 0.0075 M. The average K_v is 0.0168. The crosses indicate the course of a curve using $K_v = 0.0168$ for all points. The values of K_v at various points of the experimental curve are:

t	K_v
2	0.0228
5	0.0194
9	0.0172
13	0.0170
19	0.0158
26	0.0153
38	0.0140
53	0.0137

of t are taken, indicating a reaction accelerated at the start. However, the differences in K are not sufficient to prevent the use of an average value as an index of the speed of the reaction. By using this average any two curves may be compared with sufficient accuracy and the monomolecular formula may be thus used on an empirical basis, for convenience only and without implication that in the measurement of respiration we have to do with an actual single mono-

molecular reaction. Indeed, the fact that there is a uniform diminution in the value of K throughout an experiment is evidence that the reaction is not a simple monomolecular one (see Fig. 2).

If we assume two consecutive reactions, each with its own constant, it is possible to duplicate the experimental curves much more closely
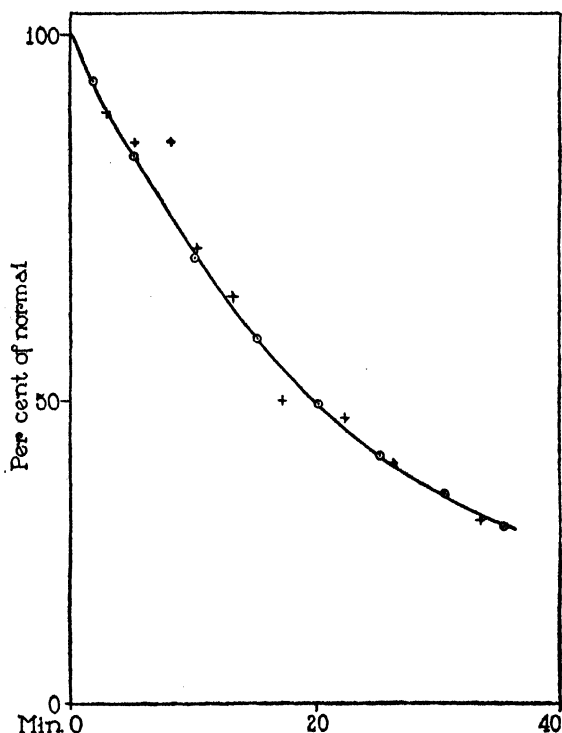
FIG. 3. Curve with copper chloride 0.02 M. $K_{\,} = 0.021$. The circles are points on a curve derived by the formula for consecutive reactions (see text), where $A = 1$, $B = 0.2714$, $K_1 = 0.0525$, and $K_2 = 0.06$. The crosses are the points obtained from the experimental data.

than by means of the formula for the single reaction. Osterhout (1922) has made this assumption with reference to similar curves obtained by the conductivity method. The mechanism of consecutive reactions will be discussed in another connection and here it will be sufficient to point out that a typical experimental curve ob-

tained with copper chloride can be duplicated (see Fig. 3), within the limits of experimental error, by using the formula for consecutive reactions. Similarly a composite curve obtained by averaging six experiments can be duplicated (see Fig. 4). Nevertheless, the simpler formula affords a good basis for comparison of curves under the influence of different conditions.
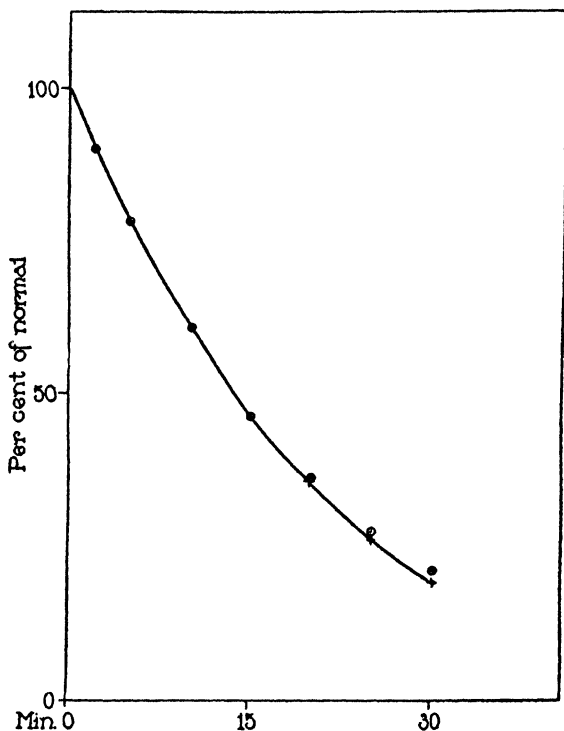
FIG. 4. The crosses are obtained by averaging six experiments with copper chloride 0.02 M. The circles are points on a curve derived by the formula for consecutive reactions (see text) where $A = 1, B = 0.2, K_1 = 0.0525, K_2 = 0.06$. The latent period is omitted.

The second metal used was mercury, as mercuric chloride. This element affects the respiration very much as does copper save that there is no latent period. The characteristic curve shows a steady drop (see Fig. 5). The monomolecular formula may be applied and
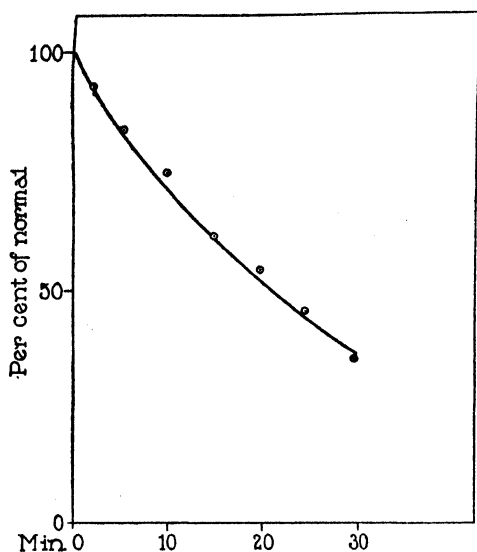
FIG. 5. Curve with mercuric chloride 0.0002 M K_v = 0.012.
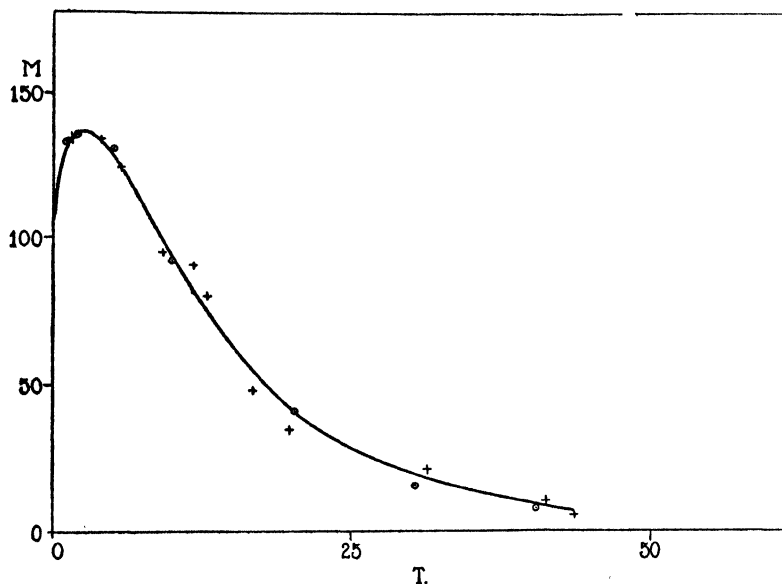
FIG. 6. Crosses indicate an experimental curve with silver nitrate 0.0001 M. K_v = 0.03.   The circles are points on a curve derived by the formula for consecutive reactions (see text) where A = 1.3, B = 2.333, K_1 = 0.12, and K_2 = 0.16.

an average constant obtained. The formula for consecutive reactions may also be applied and the experimental curves duplicated.

Silver was used as silver nitrate. The curve showed an increase in the production of carbon dioxide followed by a decrease. Although the formula for a monomolecular reaction cannot be strictly applied to such a curve (see Fig. 6), yet a constant can be obtained by letting a equal either the maximum attained or the point where the descending limb crosses the 100 per cent line. Either method is purely arbitrary yet if used consistently will give comparable results, and as in the previous case will be a useful index of the speed of the toxic action. In this connection it should be noted that the formula for consecutive reactions will duplicate the curve of silver action, as in the case of copper and mercury (see Fig. 6). Although the curve with silver cannot by any means express a simple monomolecular reaction (for the latter cannot rise above 100 per cent), it can express the result of two consecutive reactions with different velocity constants.

In connection with the heavy metals experiments were performed with certain acids. The whole molecules seem to have considerable influence in the case of some of the aromatic organic acids such as salicylic, which proved to be highly toxic in a concentration of 0.001 M and which is practically undissociated. But the activity of acids of the mineral and aliphatic categories seemed to depend on ionization. Hydrochloric and nitric acids had the greatest effect while sulfuric was less active in the same molar concentration. In the case of HCl, H_2SO_4, H_3PO_4, trichloracetic, and tartaric acids the effect of each was approximately proportional to the hydrogen ion concentration and not to the molecular concentration. Solutions of each at about the same pH value gave about the same velocity constant (see Fig. 7). The pH values were calculated from the molecular concentrations and the dissociation constants as obtained from Clark (1922).[1] With low concentrations of hydrochloric acid, which are known to be almost completely dissociated, the experimental curves were almost identical with those for copper and for mercury, and both the simple and consecutive reaction formulas will apply as in the other cases (see Fig. 8). Furthermore, the relation to concentra-

[1] Clark (1922), p. 462.

tion is the same. These results render it probable that hydrogen in the ionic form exerts a toxic action similar in nature to that of the heavy metals. If this assumption be made, then hydrogen may be classed with the metals and its action compared with theirs in the present investigation. If it is assumed that hydrogen acts in combina-
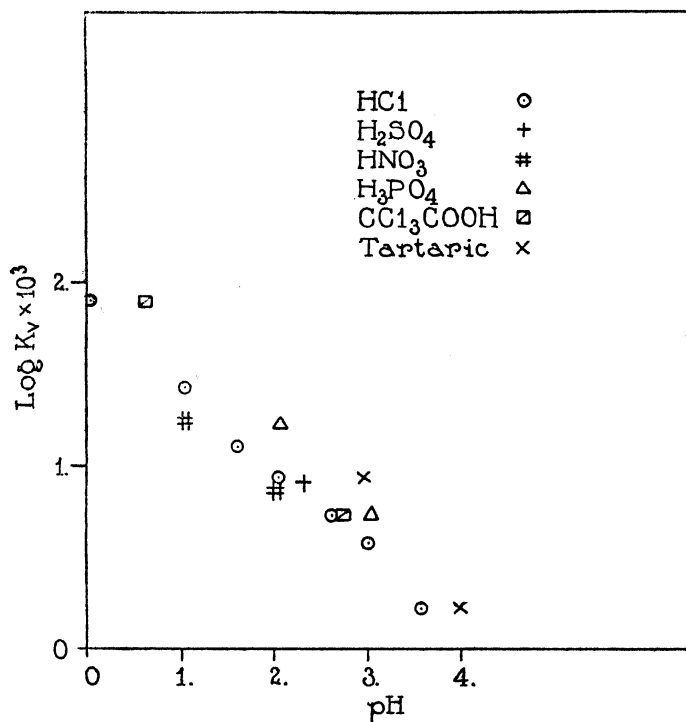
FIG. 7. The pH values of various acids plotted against the velocity constant.

tion then the comparison may still be made, for we may regard hydrogen chloride as comparable with cupric and mercuric chlorides and with silver nitrate.

IV.

We have next to inquire what is the effect on these curves of changes in temperature and concentration. Variations due to temperature were studied in the case of copper alone, but there is no
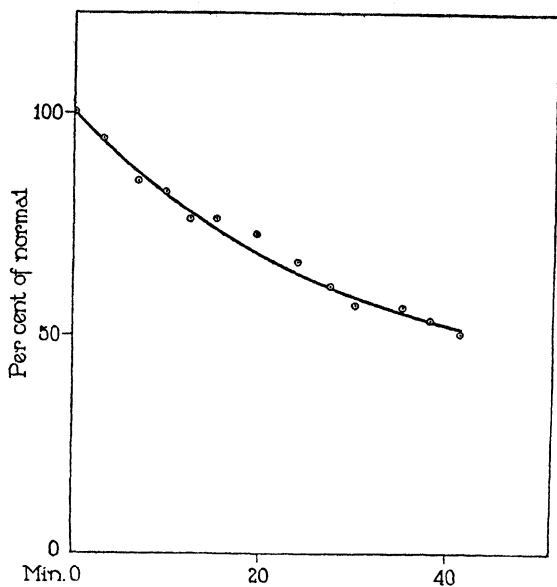
FIG. 8. Curve with hydrochloric acid 0.01 M.
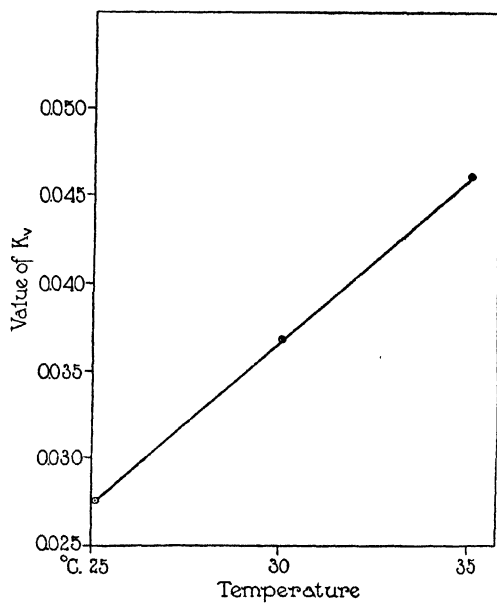
FIG. 9. The relation of temperature to the velocity constant of the action of copper chloride.

reason to suppose that it affects the action of the other elements differently. The concentration effect was determined for each of the four elements.

The temperature of the respiration chamber was varied by means of the thermostat and several experiments were performed with copper chloride, keeping the concentration constant. The temperatures were then plotted as ordinates against the value of the velocity constants as abscissæ. The result (see Fig. 9) showed that the temperature coefficient of the toxic action was about 1.8 for this range of
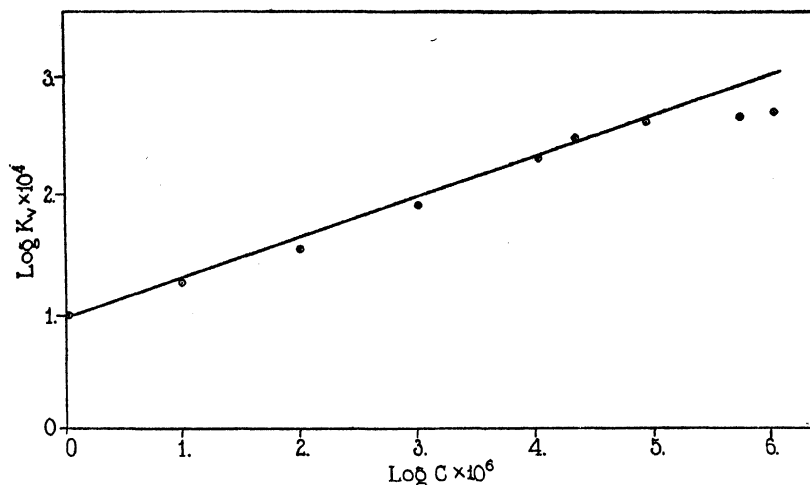
Fig. 10. The relation of the logarithm of the concentration to the logarithm of the velocity constant with copper chloride.

temperature (25–35°) at least. This is of the order commonly met with in chemical reactions and seems to exclude physical phenomena which usually have much lower coefficients but the work on temperature has been carried only far enough to determine the general order of the Q_{10} coefficient. The recent results of Crozier (1924–25) indicate that much further investigation would be profitable in this connection.

In studying the effect of concentration a range of 0.1 M to 0.000,001 M was investigated. With the other elements the concentrations lay between these values, but did not necessarily reach the extremes.

If, with any one element, the velocity constants for the curves are plotted against the concentrations as abscissæ the resulting curve is an hyperbola. When the logarithms are plotted the points fall very nearly on a straight line. In the case of copper, when high concentrations are reached the experimental line bends and tends to approach the horizontal (see Fig. 10). With the exception of this deviation, which will be again referred to, the relation may be expressed as follows: if v is the velocity, c the concentration, and b a constant, then log v is proportional to log c and $v = c^b$. This is the well known power relation which has been derived biologically a number of times (Chick, 1908; Paul, Birstein, and Reuss, 1910) in work on the effect of toxic substances.

<div style="text-align:center">V.</div>

The generally accepted theory of cell respiration is that the carbon-containing substances are oxidized by the instrumentality of catalysts. Opinion differs as to the nature of these catalysts and their mode of action. For present purposes, and until toxic action shall be better understood, it is necessary to assume merely that respiration occurs in a series of consecutive reactions, each step being catalyzed by a different substance or by the same substance in different states. All the seriously considered hypotheses make some such assumption.

The time honored theory of Engler and Bach states that oxygen is activated and handed on, as it were, by means of oxidases. The molecular oxygen is first brought into combination with an autoxidizable substance and by means of the energy furnished by this reaction forms also a peroxide. The active oxygen of the latter is handed on from one substance to another until it forms carbon dioxide, as the final number of a series of compounds thus: O_2, AO_2, BO_2,—CO_2. The precise nature of the intermediate compounds has never been discovered, nor indeed has much of the mechanism been made clear, save that it is controlled by the oxidizing enzymes and proceeds by a series of consecutive reactions.

Wieland (1912, 1913, 1914) has developed the idea that the essential process in oxidation is not the activation of oxygen but of hydrogen. Thus a substance is not oxidized by the addition of oxygen but by the removal of hydrogen, the latter if necessary being fur-

nished by water. The pertinent enzymes are called by him dehydrogenases, and by Thunberg (1920, 1921) hydrogen transportases. According to this theory a series of reactions involving hydrogen can be constructed thus: $AH_3 \rightarrow BH_2 \rightarrow CH_2 \rightarrow \rightarrow D$.

According to Hopkins (1921, 1922, and Meyerhof (1924) the activator of cell oxidations is some substance containing the sulfhydril group $HS - SH$ (glutathione of Hopkins, cysteine, thioglycollic acid, etc.). The essential feature of this system involves the alternate addition and removal of hydrogen. This in a way depends on dehydrogenation and is effective through a series of consecutive reactions.

Warburg (1921, 1925) has recently developed the idea of iron as the active catalyst. According to him the sulfhydril group is present but plays a subordinate rôle, and oxygen, not hydrogen, is activated. His scheme is of this sort: Bivalent iron + oxygen→trivalent iron + organic material→bivalent iron + oxidation products. This involves the alternate oxidation and reduction of iron and proceeds likewise though a series of consecutive reactions.

We may then assume that respiration does take place in successive steps, each step under the influence of a catalyzer, and we may draw up a conventional representation of the process as follows: $A \xrightarrow{K_1} X \xrightarrow{K_2} Y \rightarrow Z$ where A represents the initial substance, Z the end-products, and X and Y intermediate stages.

To explain the mechanism of the toxic action of the heavy metals and of hydrogen the hypothesis is advanced that these substances alter the velocity constants of these reactions, either increasing or decreasing them. Mellor (1904) and Osterhout (1922) have given analyses of such reaction systems. If we have the substance X which is being converted into Y at a constant rate K_1, and Y is being converted into Z at a constant rate K_2, then the quantity of Y present will always be constant. The substance X is being replaced as fast as it is decomposed, from a precursor A. If any factor enters the system which changes the rates of formation of X and Y and Z the quantities of each component will change. For instance let the reaction $A \rightarrow X$ stop and let K_1 be increased. Then Y will increase because it is being formed faster than it is broken down. At the

same time X is being rapidly decomposed because it is no longer being formed from A. After it sinks below a certain level it will be unable to form Y as fast as Y is decomposed and, therefore, Y will decrease from this point on. Instead of increasing K_1, we may increase K_2. Then Y will be decomposed faster than it is formed and will decrease from the start. If both the constants are multiplied by the same factor the speed will be greater but the shape of the time curve of Y will be the same except that all the abscissæ will be divided by this factor.

Stated as a differential equation, $\dfrac{dY}{dt} = \dfrac{dX}{dt} - \dfrac{dZ}{dt} = K_1 X - K_2 Y$. This may be integrated so that the quantity Y present at any time t will be expressed thus: $Y = A\,(e^{-K_2 t}) + B\left(\dfrac{K_1}{K_2 - K_1}\right)(e^{-K_1 t} - e^{-K_2 t})$.

In this form of the integrated equation K_1 and K_2 are the velocity constants as explained above, A represents the amount of Y present when the equilibrium is disturbed by the toxic agent, and B represents the amount of X at the same moment. This is the previously mentioned formula for consecutive reactions, by the use of which the experimental curves may be duplicated. In the case of the cell, Y would represent the catalyst which activates directly the formation of carbon dioxide and when we measure the rate of production of carbon dioxide we have a direct index of the quantity of Y present. In other words, when a metal interferes with the system $X \xrightarrow{K_1} Y \xrightarrow{K_2} Z$ so as to alter K_1 and K_2, the quantity of Y changes according to the formula and the observed rate of carbon dioxide production changes in an identical manner.

One point of fundamental importance in this theory is that the metals are thought of as either inhibiting or accelerating the reactions governing the formation of the catalysts. If the metal promotes the formation of the catalyst it has an effect which might be compared to the so called *promoter action*. In speaking of catalysis by iron W. C. McC. Lewis (1923)[2] says, "The presence of a mere trace of

[2] Lewis (1923,) vol. i, p. 479.

certain oxides and salts . . . . greatly enhances the activity of the catalyst proper. These are usually spoken of as promoters." Referring to the oxidation of ammonia to nitric acid he also says,[3] "Recently iron has been employed as a catalyst in this process. The activity of iron is greatly enhanced by promoters such as cerium, thorium, bismuth, tungsten, or copper." The latter statement is particularly suggestive in the light of Warburg's theory of iron as the respiratory catalyst. The promoter action might be brought about in various ways, but for our present purpose we may regard it as due to the change in the quantity of the catalyzer Y. This in turn is caused by the action of the toxic agent in altering the velocity constants K_1 and K_2.

<div style="text-align:center">VI.</div>

A further extension of this hypothesis is necessary to account for the concentration effect of the metals. In the system $A \rightarrow X \xrightarrow{K_1} Y \xrightarrow{K_2} Z$ at the same time that K_1 and K_2 are altered by the metal we have assumed that the reaction $A \rightarrow X$ ceases altogether. The other two stages then continue and the decrease of Y usually resembles a monomolecular reaction inhibited or accelerated at the start, having the apparent velocity constant K_v. It is this apparent velocity constant which appears in the experimental data. It will be directly proportional to the amount of heavy metal present in active form and this renders it necessary to establish the relation between the metal in active form and the metal in the toxic solution as first applied to the cell. It may be assumed that after the metal, for instance copper, enters the cell, regardless of whether it penetrates in the ionic or in the molecular form, it encounters a substance which we may call T. With this substance the copper must combine chemically to form CuT if it is to alter the velocity constants of the reactions $X \rightarrow Y \rightarrow Z$. In other words, the copper must itself be activated before it can exert its characteristic toxic effect. We may note that according to Warburg iron must be activated in order to catalyze oxidation. The suggestion might be made that the activation of copper consists in a change of valence instead of a chemical combina-

[3] Lewis (1923), vol. i, p. 480.

tion, but the kinetics of the toxic action are more easily treated on the latter basis than on the former.

It is justifiable to assume that the combination of copper with T is reversible. Indeed, most chemical reactions which are homogeneous, *i.e.* which do not involve a change of state, are reversible (Lewis, 1923).[4] The reaction may be expressed in its most general form thus: $Cu + T \rightleftharpoons CuT$, but the concentration effect depends on the proportions of the components of the reaction.

Mention has been made previously of the fact that if, in the case of copper chloride, the concentration be plotted against the velocity constant (as derived from the monomolecular formula) the resulting curve is hyperbolic, and therefore if the logarithms be plotted the result will be a straight line, for all except the highest concentrations (see Fig. 10). We may ignore these highest concentrations for the present. Expressed mathematically $K_v = AC^B$ and $\log K_v = B \log C + \log A$. Log A will be equal to the distance from the origin to the point where the line would cross the 0 ordinate if the line were so far extended. We shall see that on theoretical grounds we are not justified in so extending it, but for the present log A may be retained in the formula.

This equation has been derived by Chick (1908) and by Paul, Birstein, and Reuss (1910), and has been employed in various forms by investigators who have used other methods (Ikeda, 1897; Krönig and Paul, 1897; etc.). It seems, therefore, to have a general application to the biological effects of concentration. A numerical value can be obtained for both the constants A and B when two or more concentrations and their velocity constants are known.

$$\begin{aligned}
\log K_1 - \log A &= B \log C_1 \\
- \quad \log K_2 - \log A &= B \log C_2 \\
\hline
(\log K_1 - \log K_2) &= B \ (\log C_1 - \log C_2)
\end{aligned}$$

or

$$B = \frac{\log K_1 - \log K_2}{\log C_1 - \log C_2}.$$

This value of B may be substituted and the value of A obtained. Using this method the value of B in the case of copper is 0.4.

[4] Lewis (1923), vol. i, p. 120 ff.

Referring now to Fig. 11 it will be seen that similar concentration curves can be obtained from the experimental data for mercury, silver, and hydrogen. This suggests that the action of all these elements may be subject to the same laws and that the general formula $K_v = AC^B$ may hold in all cases. A striking feature, furthermore, is that the slope of the logarithmic curves appears to be the same for each ion within the limits of experimental error. The exponent B expresses the slope of the curve, and if the slope is the same B is the same.
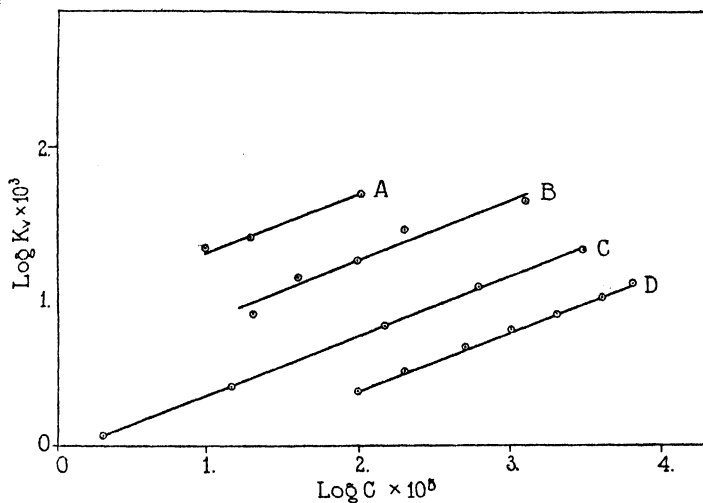
FIG. 11. The relation of the logarithm of the concentration to the logarithm of the velocity constant for: (A) $AgNO_3$, (B) $HgCl_2$, (C) $CuCl_2$, (D) HCl.

Having reached this point in the analysis of the experimental data it is appropriate to return to the hypothetical reversible reaction and to see if by this method the experimental data can be explained or at least duplicated. Consider the case of copper and the reaction Cu + $T \rightleftarrows CuT$. Since nothing is actually known of the substances entering into this reaction, it is permissible to assume any molecular proportions or any formulas for these substances that we wish. Certainly the substance T must be organic in nature and more or less complex. If we assume that the reaction runs as follows:

$$10(CuR)_5 + 2 (TV)_{25} \rightleftarrows 25 (CuTRV)_2,$$
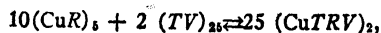
we can treat it as an ordinary reaction. R and V are used in combination with Cu and T because Cu_5 and T_{25} could scarcely exist alone as polyatomic ions but R and V are entirely neutral as far as the reaction is concerned. This point being understood we can write for simplicity: 10 Cu_5 + 2 $T_{25} \rightleftarrows 25$ $(CuT)_2$. Now if the reaction proceeds to equilibrium, the equilibrium constant K_e will equal the ratio between the products of the reacting molecules on each side of the equation. In the simple reaction $Cu + T \rightleftarrows CuT$ the equilibrium constant K_e will equal $\dfrac{(Cu) \ (T)}{(CuT)}$, since there is one molecule each of Cu and T on the left side and one molecule of CuT on the right side. In the special case above we can divide the coefficients of both sides by the same number, and if we so divide by 5 we obtain the following equation: 2 Cu_5 + $0.4 T_{25} \rightleftarrows 5$ $(CuT)_2$. When this is in equilibrium $K_e = \dfrac{(Cu_5)^2 (T_{25})^{0.4}}{(CuT)_2^5}$. Now if p be the number of molecules of Cu_5 at the beginning of the reaction, q the number of molecules of T_{25}, and at equilibrium r is the number of molecules of $(CuT)_2$, then at equilibrium the number of molecules of Cu_5 is equal to $(p - r)$ and of T_{25} $(q - r)$. We may now write $K_e = \dfrac{(p-r)^2 (q-r)^{0.4}}{r^5}$. By rearranging and putting into the logarithmic form we obtain:

$$\log (p - r) \ \frac{\log K_e + 5 \log r - 0.4 \log (q - r)}{2}.$$

If we assume values for p, q, and K_e we can calculate the value of r, In this manner the following table is obtained ($K_e = 0.016$):

p	q	r
0.1001	1000	0.1
1.03	"	1
21	"	10
188	"	30
366	"	40
960	"	60
3546	"	100
19050	"	200
114000	"	400
742000	"	800
6313000	"	999.9

If the logarithm of p is plotted against the logarithm of r, Fig. 12, Curve B is obtained. This curve comes reasonably close to coinciding with the experimental curve for copper in the same range of concentration. If the proportions of the reacting substances were assumed with greater exactness a much more accurate duplication would be the result.

Regardless of the precise numerical value of the exponent, it is apparent that the relation is: $r = ap^b$ in which a is a constant. This is just the relation found where $K_v = AC^B$ as determined experimen-
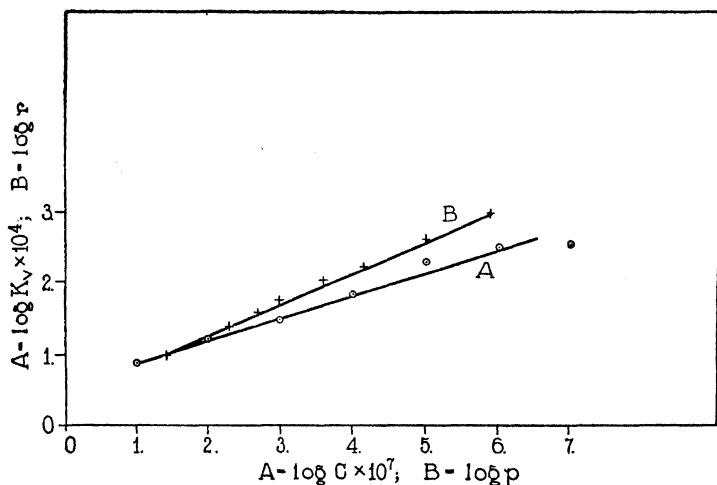
FIG. 12. Circles indicate the concentration effect with copper (*cf.* the middle portion of Fig. 10). The crosses are obtained by the use of the formula:

$$\log (p-r) = \frac{\log K_e + 5 \log r - 0.4 \log (q-r)}{2}.$$

tally. K_v corresponds to r, A to a, C to p, and B to b. The exponents B and b differ slightly, as explained above, because the assumed values in the calculation did not exactly fit the experimental case. In the experimental curve C represents the actual external concentration of the copper, in the calculated curve it represents the same thing. In the experimental curve K_v represents the speed of the toxic action; in the calculated curve r represents the amount of the compound CuT present at equilibrium. But it has been pointed out that the speed of the toxic action represented by K_v, or the change

in the rate of carbon dioxide production, is directly dependent on the amount of active copper (CuT) present. Thus although these considerations cannot be said to prove the hypothesis of a reversible reaction, nevertheless by that hypothesis the experimental concentration effect can be duplicated.

In the experimental curves of the metals and of hydrogen (see Fig. 11) it will be observed, as already noted, that the relation between $\log K_v$ and $\log C$ for each one appears to be linear, and that the lines are parallel. This indicates that the exponent B is the same in all cases but the coefficient A is different. That is to say, the lines if prolonged backward apparently would cross the 0 abscissa at various distances from the 0 ordinate. Furthermore, if prolonged forward they apparently would continue indefinitely without change of slope. There is no direct experimental evidence concerning the extensions of these curves and, this being the case, we are not justified in extrapolating the curves beyond the points actually obtained by experiment. If the lines should not cross the 0 abscissa in the manner we have assumed in evolving the formula $K_v = AC^B$, then we have no right to use the coefficient A except empirically, and then only in treating those portions of the curves which are furnished by the experimental data. In the calculated curve

$$K_c = \frac{(p - r)^2 (q - r)^{0.4}}{r^5},$$

or

$$\log (p - r) = \frac{\log K_c + 5 \log r - 0.4 \log (q - r)}{2}.$$

Now suppose we let r become smaller. Then r tends to approach the value of p as shown in the table (e.g. when $r = 0.1$, $p = 0.1001$) and in Fig. 13, Curve B where these points are plotted. The solid lines of the experimental region are extended as dotted lines backward past the intersection of the ordinates. If, on the other hand, r approaches q the curve will bend over, as indicated by the dotted line to the right in Fig. 13, Curve B, and p will become indefinitely large. Thus since the quantity of q is always the same, the quantity of r can never exceed it, no matter how great the concentration of p. That a similar situation exists experimentally is shown in Fig. 10,

where the points tend to approach a horizontal line at the right of the curve. It was pointed out previously that with the higher concentrations of copper the toxic effect ceased to increase rapidly with increase in concentration. We can here account for the phenomenon by saying that there is a definite amount of T (represented by $q = 1000$) in the cell which limits the amount of CuT which can be formed with increasing concentrations of copper. It is evident that we
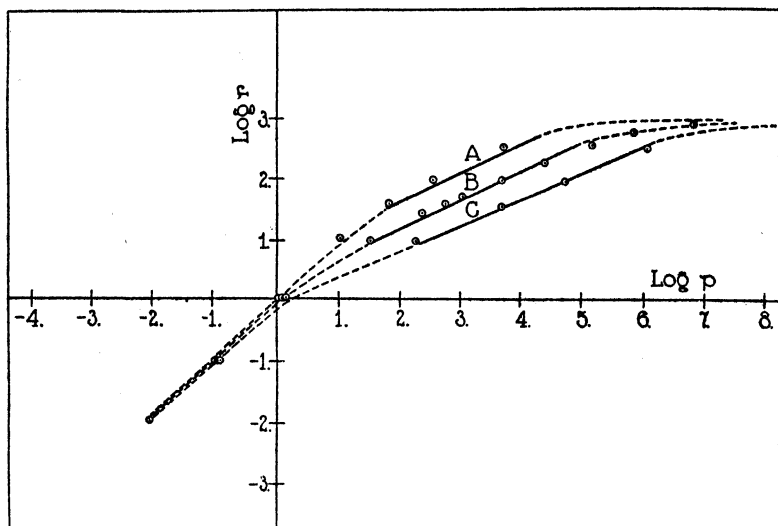
FIG. 13. Curves A, B, and C are calculated by means of the formula:
$$\log (p-r) = \frac{\log K_e + 5 \log r - 0.4 \log (q-r)}{2}$$
where $K_v = 0.00004$, 0.016, and 2.5 respectively. Curve B is the same as in Fig. 12, Curve B. The solid lines represent the middle portions of the curves such as are obtained experimentally. The dotted portions represent the theoretical continuations of the curves when r approaches p and q.

should not be justified in extrapolating the graphs in Fig. 13 to the right as continuations of the straight lines.

Returning to the calculated curves in Fig. 13, the dotted portion to the left makes it clear that since log p and log r both approach minus infinity the curve will not cross the 0 ordinate at any such distance (log A) from the 0 abscissa as a continuation of the experimental curve would lead us to suppose. It will actually cross below

the intersection of the ordinates. It is apparent from an inspection of the experimental and calculated curves that the experimental data give us the middle portion of the entire curve and that the middle portion approximates a straight line. For this portion, therefore, the relation $K_v = AC^B$ holds, and we may use it, as we use the formula for the monomolecular reaction, as sufficient working index to the concentration effect. But we cannot consider it to be the precise relation throughout the entire range of concentrations from zero to infinity. The exponent B will differ at very small and at very great concentrations, and since the value of A represents an extension of the straight line which does not really occur, the simple power relation can be of value only empirically and with a limited range of concentrations.

The experimental curves with the other metals (Fig. 11) likewise appear to be straight lines. But we know from the case of copper that their continuations will not be straight. Probably the experimental data have given a portion of each which is approximately straight. However, the fact that they do not coincide in this range of concentrations shows that they differ to the extent that for a given concentration the toxic effect of some is greater than that of others. On the basis of the experimental curves alone this variation in toxicity would be referred to differences in log A. This would provide a working basis for the comparison of the elements, but it would furnish no clue to the nature of the differences between them. And on theoretical grounds log A is an unsafe criterion since the use of it depends on an arbitrary extrapolation of the curves. A sounder criterion is the equilibrium constant of the reaction $M + T \rightleftharpoons MT$ in which M stands for any cation.

Since the curves show that for a given concentration the toxic effect of the elements varies, we can take this to mean that more MT is formed by some than by others. In order for more MT to be formed by the same concentration of M the ratio $\dfrac{(M)\,(T)}{(MT)}$ must be smaller. This means a difference in the equilibrium constants of the reaction. There is no method for determining the equilibrium constants of the experimental curves directly, but we may start with the calculated curve for copper (Fig. 12, Curve B) and try to duplicate the curves

of the other metals. In Fig. 13 there are three curves calculated by the usual formula using K_e = 0.00004, 0.016, and 2.5. It was previously pointed out that the middle portions of each (solid lines) are approximately straight lines and are parallel, whereas the extremes (dotted lines) are unquestionably curved and converge as p approaches zero and infinity. If we had only the middle portions we
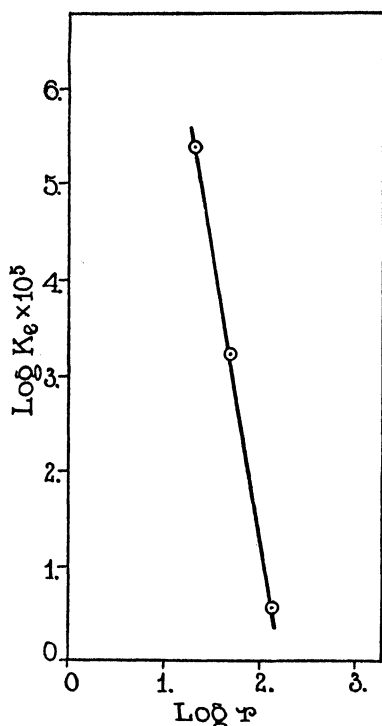
FIG. 14. Points obtained from the curves of Fig. 13 when log p = 3. Log K_e × 10^5 is plotted against log r when log p is thus fixed.

should be very close to the experimental curves for mercury, copper, and hydrogen, and should consider the calculated curves to be straight lines.

If we consider, in the calculated curves, a given concentration, say that represented by 1000 (log 1000 = 3 on the curve), and plot the logarithm of r against the logarithm of K_e, we obtain Fig. 14.

Apparently here also is a power function, since the relation of the logarithms appears to be inversely linear. Then

$$\log K_e = -n \log r, \quad \text{or} \quad K_e = r^{-n}.$$

If we take different values for r:

$$\log K_{e1} = -n \log r_1$$
$$- \qquad \log K_{e2} = -n \log r_2$$
$$\overline{(\log K_{e1} - \log K_{e2}) = -n (\log r_1 - \log r_2)}$$

or

$$\left(\frac{K_{e1}}{K_{e2}}\right) = \left(\frac{r_1}{r_2}\right)^{-n} \quad \text{or} \quad \left(\frac{K_{e2}}{K_{e1}}\right) = \left(\frac{r_2}{r_1}\right)^{-n}.$$

Now r in the calculation is equivalent to MT, or the amount of active metal, and this in turn is proportional to the speed of the toxic action K_v. Therefore we may substitute K_v for r and write:

$$\left(\frac{K_{e2}}{K_{e1}}\right) = \left(\frac{K_{v2}}{K_{v1}}\right)^{-n} \quad \text{or} \quad K_{e2} = \left(\frac{K_{v2}}{K_{v1}}\right)^{-n} \times K_{e1}.$$

In the original calculated curve of copper (Fig. 12, Curve B, and Fig. 13, Curve B) the constant $K_e = 0.016$ was assumed. We now wish to find K_e for mercury, using the experimental data. This we may do for any concentration, say 0.001 M. Making the appropriate substitutions for K_{v1} and K_{v2}, from actual experiments, we get $K_{e2} = \left(\dfrac{0.028}{0.0085}\right)^{-n} \times 0.016$. If we let $n = 5$ and solve, we get $K_{e2} = 0.00005$ where the calculated value was 0.00004 (Fig. 13, Curve A). Similarly, using the experimental data for hydrogen, $K_{e2} = 1.39$ where the value used in calculating Fig. 13, Curve C, was 2.5. These values are fairly close though not as close as might have been obtained had the experimental curves been exactly duplicated by the calculated ones. They are, however, of the same order of magnitude. Furthermore the relation $K_e = r^{-n}$, as with the relation $K_v = AC^B$, cannot hold with very small or with very great values of r. It holds empirically, however, for the middle ranges of concentrations which are subject to experimental analysis. This fact being granted, we may use this formula, as well as the other, in analyzing experimental data.

VII.

Any hypothesis must account for as many as possible of the observed facts. It is believed that in this instance the facts may be explained by assuming a reversible reaction which activates the toxic elements (Cu, Ag, Hg, H) and enables them to inhibit or destroy in a characteristic manner the normal oxidation catalysts. The various phenomena observed in connection with this action are explicable on the assumption of appropriate reaction velocities. This hypothesis is advanced solely as a working model and no claim is made for its ultimate truth. However, it provides a convenient and rational way in which to correlate a large number of otherwise isolated and unrelated facts. An attempt has been made to put all relations on a purely chemical basis. This has been done deliberately and in the light of the knowledge that alternative theories might well be advanced. For instance, many of the phenomena here recorded could be treated from the point of view of surface action or adsorption and some of the curves duplicated; notably, the curve of concentration effect. But it is the opinion of the writer that an explanation from a purely chemical point of view accounts for more of the results and is in general more satisfactory.

SUMMARY.

1. The effect of the heavy metals on the respiration of *Aspergillus niger* is to cause the rate of carbon dioxide production to decrease from the first or to increase and subsequently diminish.

2. The speed of the toxic action varies as a constant power of the concentration.

3. The temperature coefficient of the toxic action is between 1.5 and 2.

4. An hypothesis is advanced to account for the action of the heavy metals, by means of which the experimental results may be accounted for. It is assumed that the metal is activated by a chemical combination with a cell constituent. This active compound alters the velocity constants of the normal respiratory reactions, and thus causes the observed changes in the rate of carbon dioxide production.

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PHYSIOLOGICAL ONTOGENY.

A. CHICKEN EMBRYOS.

VIII. ACCELERATIONS OF INTEGRATION AND DIFFERENTIATION DURING THE EMBRYONIC PERIOD.

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PLATES 4 AND 5.

It has been shown by many biologists that with age there is a decrease in the percentage growth rate, a differentiation of structure and function, and a slowing of metabolism. As a consequence it seems to be generally assumed that these processes are concomitant and that the changes with age of their respective velocities are parallel. The results of recent studies of the physiological ontogeny of chicken embryos have demonstrated the error in this assumption, and have shown the necessity for the sake of precision of introducing certain distinctions and qualifications in the accepted concepts. The phenomena primarily examined were (1) growth, (2) gross form development, (3) concentration of solids, and (4) chemical differentiation. It was found that there were significant phase differences among these; *i.e.*, differences in the period of incubation when changes were rapid or the reverse. The rates of the first two functions (growth and form) changed rapidly in the beginning, whereas the latter (concentration and differentiation) changed mostly during the last half of incubation. With the period of maximum rate change as a criterion, two type rate-curves might be described, types A and B respectively, which in this particular are opposite to one another. They seem to show skew symmetry around a central point. This phase difference may be used as a basis for distinguishing between primary or gross integration

(growth) which occurs more or less synchronously with primary or gross differentiation of form,—type A curve; and secondary or internal integration (concentration of solids) which is concomitant with internal differentiation of chemical form,—type B curve. These groups are comparable to Spencer's division of the evolutionary process into primary and secondary redistributions or so called simple and compound evolution. In the present communication it is pointed out that rates of these ontogenetic processes change most markedly at different times during the life span.

Treating the body as a whole, the gathering together and storage of matter seems to be always accompanied by a change in its outward form; and similarly in the case of the internal composition of the body, the increasing concentration of matter is found associated with a concomitant change in chemical complexity. Instead of introducing new terms in this communication we shall use *growth* to signify primary or gross integration, *i.e.* the increase in total weight; the term *integration* itself to signify the increasing concentration of solid substance within the organism; *form* to describe the gross structural relationships of the body as a whole and its organs; and, finally, *differentiation* to describe progressive changes in microscopic form and chemical constitution. The expression *growth rate* refers to the percentage rather than the increment rate of growth.

The experiments to be described were all done on one species over one period of its life cycle; namely, the chicken embryo between the 5th and 19th days of incubation. No assertion is made in regard to the applicability of the relations discovered to other species or to the hen over any other period of its life cycle.

Growth Rate.

The growth rate of the chicken embryo exclusive of membranes has already been measured with some care under certain conditions of incubation (temperature 38.8°C.; humidity 67.5 per cent) which we arbitrarily selected as standard (1). It was found that between the 5th and 19th days of incubation, the percentage growth rate decreased progressively with age. In seeking to explain this, or at least in seeking for certain physical and chemical conditions which might be correlated with, and hence be said to account for the change in the

growth rate, attention was at first directed to two well known hypotheses; (1) that dealing with the surface/volume ratio, and (2) Robertson's autocatalytic theory (2).

The autocatalytic concept of growth has been accepted by many biologists within recent years and has formed the theoretical basis for much profitable investigation. Relatively simple formulæ have been found to fit values empirically determined. There are, however, a number of objections to this theory, some of which should be enumerated.[1]

(1) The formula demands the introduction of three different constants, which must be separately determined for every set of figures collected. (2) The equation does not give the weight as a function of age throughout life, but only during an arbitrarily selected part of the growth cycle. For these two reasons the equation as a practical simplification is not of great value. If the equation were in such a form that knowing the species of organism, the age, the temperature, and other environmental variables one might calculate the weight and growth rate, it might be of use. As it stands now it is necessary in each case to collect complete statistics and then find a mathematical expression of the figures obtained. For instance all three constants in the equation for the growth of South Australian males differ from the constants used in the equation for South Australian females. As one cannot extrapolate, the formula like the man with one talent, returns what it receives. In fact as it covers only a section of the growth curve it yields less information than the original data. Moreover, as a rational description this representation of the synthetic processes of growth is misleading, since (3) by this theory the growth rate is proportional to the increment gain in weight regardless of the weight of the organism or, in other words, disregarding the amount or concentration of the reacting substances. It is not without reason that it is common practice to express quantitative biological data in terms of weight, volume, surface area, etc. (4) Figures for the growth of colonies as well as of individual organisms are said to be described by autocatalytic equations, and are classed together. When growth is expressed in terms of percentage increase in mass, however, the important distinction between phylogenetic and ontogenetic growth is made evident. Colony, racial, or phylogenetic growth (*cf.* bacteria and paramecia), after a short latent period, in the presence of an experimentally modified environment, proceeds at a *constant* rate. The S-shaped curve is the result of a limited

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[1] A correspondence with Dr. S. Brody who has written several important papers on this subject (*J. Gen. Physiol.*, 1920–21, iii, 623, 765; 1922–23, v, 205, 445; 1923–24, vi, 31, 239) and who disagrees with me in this particular matter, has helped me to formulate the objections to the autocatalytic theory which are presented here.

and unrefreshed culture medium. The individual organism, however, instead of maintaining a constant growth rate shows from the beginning considerable negative acceleration. In other words the rate of phylogenetic growth theoretically and under experimental conditions proceeds unchecked whereas ontogenetic growth continuously declines in rate.

That curve-fitting should lead to definite conclusions as to the nature of the physiological process involved is unfortunate. We believe that autocatalysis is a spurious analogy for organic growth. In so far as it is statistical rather than mechanical the population curve would make a better analogy. (5) The argument that growth is autocatalytic is based upon the S-shaped curve. The S-shaped curve, however, is not specific. There are some physicochemical processes not considered to be autocatalytic which are described by a similar curve. (6) An autocatalytic process is usually considered as one for which the law of mass action is not completely descriptive, since although the concentration of the substrate diminishes in amount, the velocity of the reaction is accelerated due to the catalytic activity of one or more of its products. In the case of the living organisms there is no necessary limit to the food supply and thus nothing that really corresponds to the diminishing substrate of a chemical reaction; whereas the catalytic agent in Robertson's description would seem to be the increasing dimensions or mass of the organism itself.

Finally, and this is the main objection, (7) chemical differentiation is not taken into account by the autocatalytic theory. The autocatalytic theory is based upon the conception that there is some one master monomolecular reaction which, being the slowest of the chain of reactions concerned in the phenomenon or growth, determines the velocity of the entire process. As there is no direct way of measuring the product of the "master reaction," the increase in the total body weight is taken to represent the product. In view of the marked changes in the chemical constitution of the tissues with age, however, there is no reason to suppose, and in fact it is extremely unlikely, that the total weight can be taken as an index of the amount or concentration of any one chemical substance.

A few objections to the surface /volume theory may be mentioned here. With the increase in mass of a perfect sphere, the surface area changes as its square, the volume as its cube. This simple relationship evidently does not hold for an organism of complex form. In the first place the organism does not maintain a spherical shape with growth and secondly there are many other surfaces of unknown area as significant theoretically as the outer skin surfaces. In the case of the chicken embryo there may be mentioned the capillary surfaces of the following structures; the respiratory membrane, the yolk sac membrane, the tissues and organs throughout the body, and, once excretion commences, the renal tubules and glomeruli. Finally there are the individual cell surfaces, and the innumerable molecular and phase surfaces within the cells.

Any simple conception that growth rate depends upon the surface /volume

ratio without mention of other qualifying factors seems inadmissible for the following reasons.

(1) In the case of the individual cells of metazoa which are, as far as we know, about the same size throughout life, the average of their surface/volume ratios would not change with development any more than it would in a growing colony of unicellular organisms.

(2) The surface/volume ratio may theoretically be maintained at any level simply by the infolding or wrinkling of the surface, as is seen in the intestines. It is only when a constantly globular form is maintained that the above mentioned numerical relationship of surface and volume is imposed upon a body with growth.

(3) In actuality the area of capillary surface is adjustable since the development of new vessels such as is seen in the process of repair may occur as the result of repeated vigorous functioning of a part.

(4) It is known that, under normal conditions, only a fraction of the capillaries and therefore of an exposed surface is open or active at any one time. For instance the amount of heat radiation from the skin actually depends less upon the measured skin surface than upon vascular changes, which, in turn, depend upon the metabolic rate rather than *vice versa*.

(5) It is not only the area of the surface but the permeability of the surface that is important, and as the chemical constitution of each cell changes markedly with age, so also will its surface permeability change. It has been shown for instance that membrane permeability changes during the interdivisional period of a single cell in the metazoa (3).

(6) The hypothesis that growth is correlated with the area of absorptive surface supposes that through a given unit of surface a certain restricted number of molecules may pass per unit of time. But, if, as we know, there is a change with age in the kind and therefore size and migration rate of the molecules which enter the cell, one would hardly expect this simple relationship to be maintained.

(7) Growth or storage is the difference between absorption and elimination. Either one of these factors may vary more or less independently of the other, and thus growth is necessarily dependent upon both of them. As the ratio of storage to assimilation changes with age, if absorption is dependent upon surface, growth cannot be, and *vice versa*.

The surface of the embryo has not been measured. Assuming that $S = KW^{\frac{2}{3}}$, where S is the surface and W the weight of the embryo, and assuming, moreover, that there is no very marked change in the value of K with age, a general idea of the changes with age in the skin surface and consequently in the surface/volume ratio may be had (Table I; Fig. 1). It is probable that the general shape of the curve is about right, and despite the theoretical objections raised to the surface/volume ratio hypothesis, it is of some interest because it is the

TABLE I.

The Weight, Growth Rate, CO_2 Production Rate, and Percentage of Solid As Functions of the Age of Chicken Embryos.

Age.	Weight of embryo.	Log weight.	$W^{\frac{1}{3}}$	$W^{\frac{1}{3}}/W$ ratio.	$Log\left[\dfrac{100\,W^{\frac{1}{3}}}{W}\right].$	Growth rate.	Log (growth rate × 100).	Rate of CO_2 production per 24 hrs. per gm.	Reciprocal of CO_2 production.	Solid.	Log per cent solid.
days	mg.					per cent		cc.		per cent	
5	221	2.34	36.6	0.165	1.217	0.720	1.86			5.32	0.726
6	423	2.63	56.4	0.133	1.124	0.600	1.78	29.2	0.343	5.58	0.747
7	735	2.87	81.4	0.111	1.045	0.514	1.71	30.1	0.332	5.85	0.767
8	1,189	3.07	112.2	0.099	0.973	0.450	1.65	28.5	0.351	6.21	0.793
9	1,817	3.26	148.9	0.082	0.914	0.400	1.60	29.8	0.336	6.50	0.813
10	2,661	3.42	192.0	0.072	0.857	0.360	1.56	27.8	0.360	7.00	0.845
11	3,750	3.57	241.0	0.064	0.806	0.327	1.51	27.5	0.364	7.70	0.886
12	5,105	3.71	296.0	0.058	0.763	0.300	1.48	27.9	0.358	8.80	0.944
13	6,839	3.83	360.2	0.053	0.724	0.277	1.44	24.7	0.405	10.10	1.004
14	8,974	3.95	431.7	0.048	0.681	0.257	1.41	22.1	0.453	12.25	1.088
15	11,460	4.06	507.9	0.044	0.643	0.240	1.38	21.7	0.462	14.60	1.164
16	14,390	4.16	591.8	0.041	0.613	0.225	1.35	20.6	0.486	16.40	1.215
17	17,950	4.25	685.4	0.038	0.580	0.212	1.33	18.0	0.556	17.22	1.235
18	22,030	4.34	785.7	0.036	0.556	0.200	1.30	15.8	0.632	17.69	1.248
19	26,670	4.43	892.5	0.033	0.518	0.190	1.28	14.6	0.685	17.70	1.248

only rational curve we have obtained which in any way approximates the shape of the rate curves for growth and form development. Possibly the curve for the rate of absorption may be found to follow the S/W curve more closely, but in view of the above considerations it is unlikely that the surface area is the sole or even the chief variable modifying either metabolic rate or growth rate.
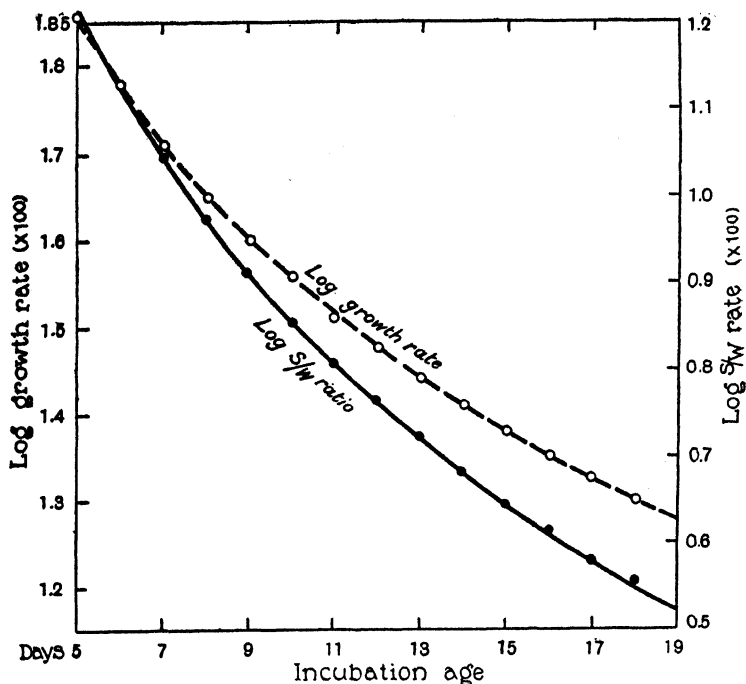
FIG. 1. The logarithm of the percentage growth rate compared with the logarithm of the surface /weight ratio as functions of the incubation age.

Form.

One who inspects the embryo at different stages of incubation will agree, it is believed, that the more marked changes in form occur in the beginning when the percentage rate of growth is rapid, and that later, when the increment growth rate increases, there is little change in outer form. This can be seen best in the heart, an organ whose growth seems to keep pace with the growth of the whole embryo.

As in the case of the total weight of the chick, the values for the projected area of the heart (4) (as measured with projectoscope and planimeter (Table II)), when expressed in logarithms and plotted against log time may be described by a straight line (Fig. 2) and the percentage growth rate of the heart area as thus drawn may be calculated and equated against age (Fig. 3). In conjunction with

TABLE II.

The Projected Surface Area of Chicken Heart As a Function of the Incubation Age.

1 Incubation age.	2 Log age.	3* Log average projected area (sq. mm. × 10).	4 $\dfrac{\frac{dA}{dT}}{A} = \dfrac{2.3}{T}$†
days			
4.8	0.681	0.689	0.479
5.8	0.763	0.947	0.397
6.8	0.832	1.136	0.338
7.8	0.892	1.095	0.295
8.8	0.944	1.315	0.261
9.8	0.991	1.453	0.235
10.8	1.033	1.390	0.213
11.8	1.072	1.515	0.195
12.8	1.107	1.709	0.180
13.8	1.140	1.720	0.167
14.8	1.170	1.850	0.155
15.8	1.199	1.855	0.145
16.8	1.225	1.882	0.137

* Figures in Column 3 obtained for the area (sq. mm. × 10) of the projected surface of the embryonic chicken heart by Cohn (4).

† Differentiation of the equation $\log A = 2.3 \log T - 0.87$ (where A is the area and T the incubation time) which was found to describe the figures given in Column 3.

these figures the microscopic drawings of the intact heart reproduced in Plates 4 and 5 should be examined. No satisfactory method of measuring changes in form quantitatively was known, so that it was necessary to resort to the expedient of selecting forms spaced by a visual impression so as to represent approximately equal degrees of gross change. In other words from a series of drawings made at

frequent intervals, certain ones were chosen which seemed by inspection to be equally spaced from one another in respect to their relative complexity of form. Thus, this test is necessarily arbitrary and open to criticism because of its subjective nature. By taking the average result of many eggs it was then determined what were the exact incubation ages of the embryos with heart forms such as those selected.
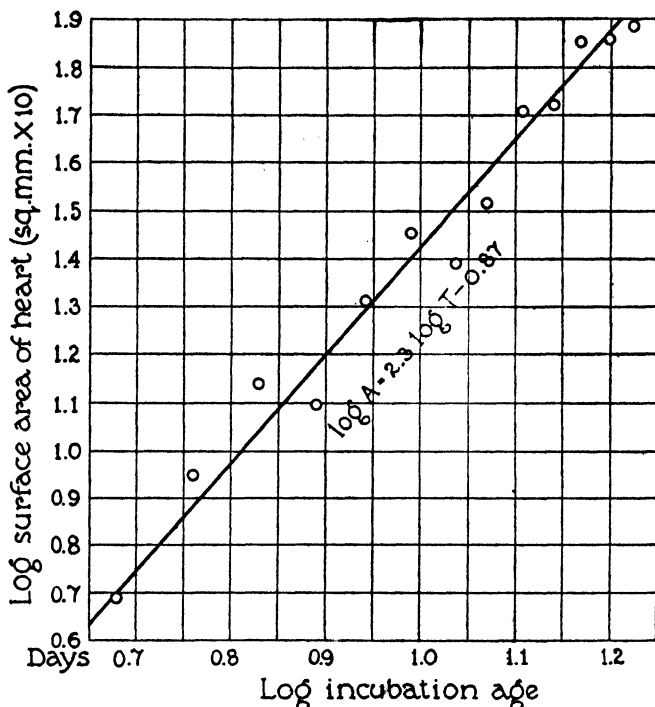
FIG. 2. The logarithm of the projected surface area of the chicken heart (in sq. mm. × 10) plotted against the logarithm of the incubation age.

The reciprocals of the time intervals between successive drawings were used as rough criteria of the rate of form development (Fig. 4). It may be seen from the figure that the rate of evolution of external form falls precipitiously at first and then ever more slowly, and thus resembles essentially the curve for the percentage growth rate. Thus growth and form seem to be aligned one with another.
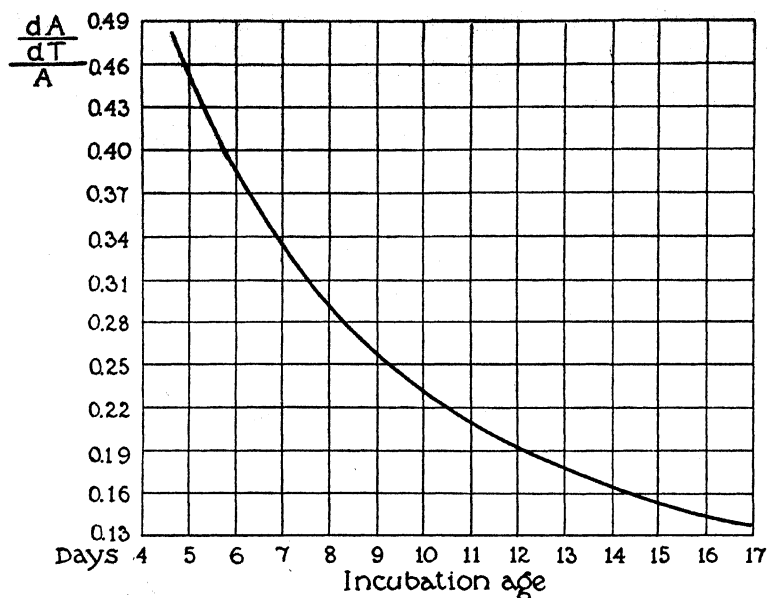
FIG. 3. The percentage growth rate of the surface area of the heart $\frac{\frac{dA}{dT}}{A}$ when Plotted against the incubation age.
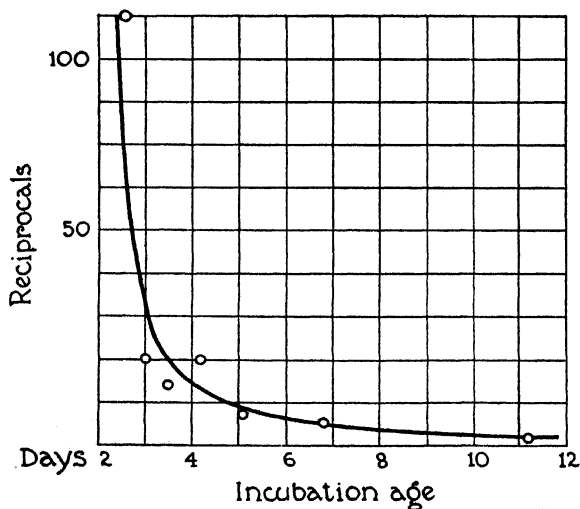
FIG. 4. This shows an attempt to represent diagrammatically the rate of gross form differentiation in the development of the heart. The ordinates are reciprocals of the times taken to make a unit change in form (*cf.* Fig. 3); the abscissæ give the incubation ages.

Internal Integration and Chemical Differentiation.

Internal integration may be regarded as a process characterized by the concentration of solid substances within the body, whereas chemical differentiation is a change in the composition of the solid
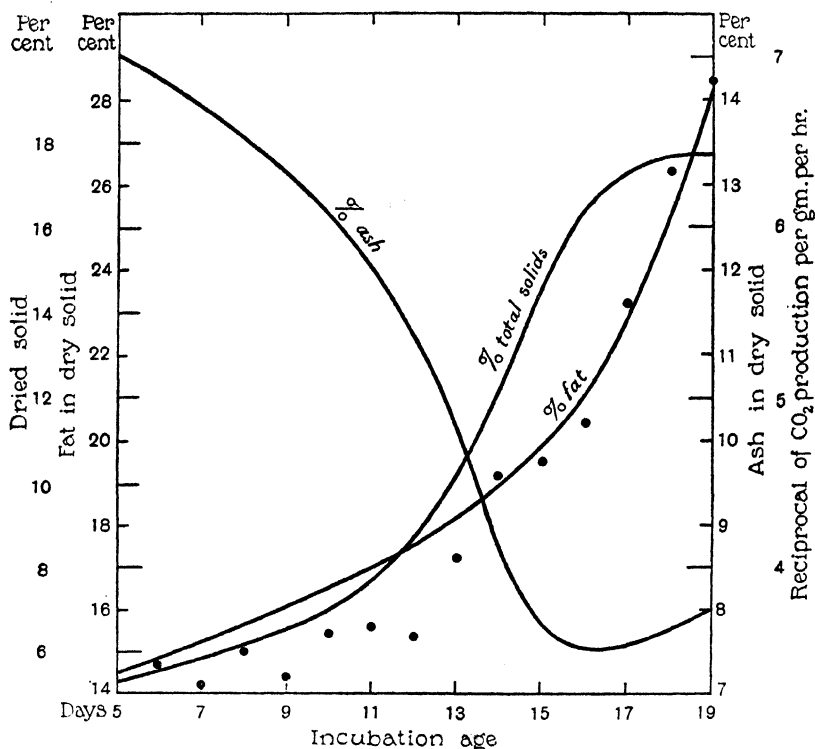
FIG. 5. The percentage of ash and fat in the dried solid, and the percentage of total solids in the whole embryo as functions of age. The solid circles (●) are the reciprocals of the rate of carbon dioxide production per gm. of embryo. The abscissæ give incubation ages.

substances thus integrated. We have no one criterion for measuring the rate of change in chemical form, but all the curves that we have obtained (5) point to the fact that the most marked changes occur after the 10th day during the last half of the incubation period. In view of the fact that all the analyses were done on the whole embryo, the correlation of the results must not be pushed too closely. It is

almost impossible to say how much the relative differences in the growth rates of the various organs will affect the relative concentrations of the different chemical constituents. For instance it is obvious that the growth of feathers and the calcification of bone will change ratios markedly, and consequently one could hardly expect to find relationships expressible by such simple equations as describe conditions in a less complex system like blood. The three curves included in the accompanying figure (Fig. 5) show the changes with age in the percentage concentration of ash, total solid, and fat (ether extract) in the chicken embryo. The ash and solid change mostly during the third and fat during the fourth quarter of the incubation period.

The curve for the percentage of dried solid in the embryo shows the changes with age in internal integration. It is more or less intermediate between the ash and fat curves and is very similar to several others to be reported which show transformations in the proportional concentration of other chemical constituents of protoplasm. Thus it seems that internal integration and chemical differentiation are more or less synchronous phenomena, and that the curve for the percentage of dried solid may be taken as an index of both. Moreover, it would seem from the theoretical considerations which call attention to the great importance of water for all chemical processes that the water/solid ratio represents as significant a function as any which one might select.

Carbon Dioxide Production.

No reliable figures for the total energy output are available as none of the determinations of CO_2 that have been made in the past were corrected for the changes in CO_2 content of the embryo and of the rest of egg contents during incubation. Thus errors have necessarily been incurred. The determinations reported in an earlier paper of this series (6), however, furnish us with approximations of some value. The reciprocals of the values for CO_2 production have been plotted as solid black circles in the accompanying graph (Fig. 5). The results point to a general correspondence, as yet mathematically undefined, between catabolic rate and chemical constitution.

Growth Rate and Latent Period in Tissue Cultures.

Distinction between two phenomena according to their changes in rate with age was demonstrated by certain tissue culture experiments previously reported (7). It was found that when parts of the heart of chicken embryos of different ages were planted in similar plasma cul-
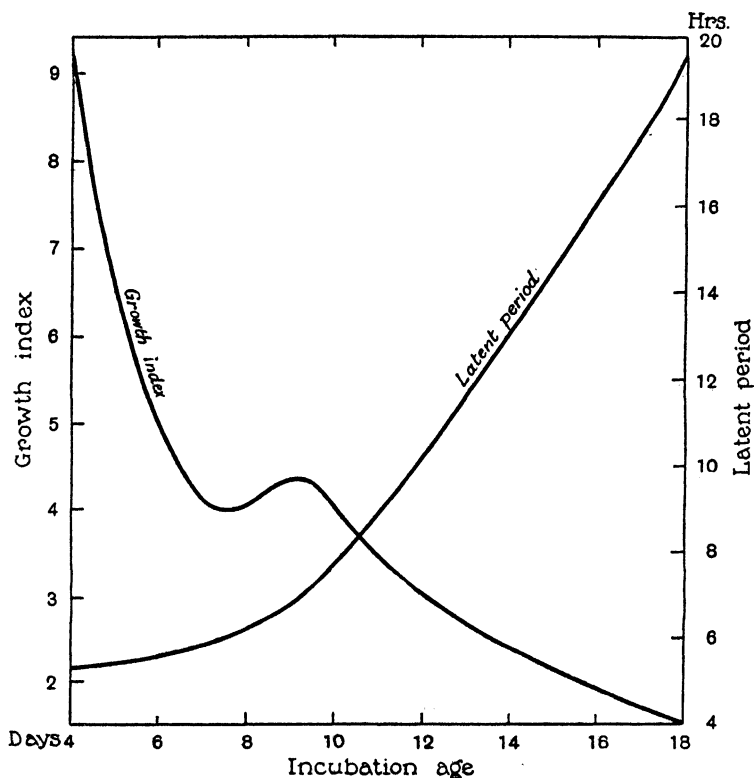
FIG. 6. The growth rate and the latent period of growth in tissue culture as functions of the incubation age of chicken embryos.

ture media the youngest pieces would not only have shorter latent periods before commencing to grow, but, when once started, growth would proceed with greater rapidity than with the older pieces. It was a surprise at the time to find that the change in the growth index was greatest in the beginning whereas the change in the latent period occurred principally during the second half of the embryonic cycle

(Fig. 6). The accompanying curves obtained from tissue culture experiments are analogous to what has been shown for the embryo as a whole, and point to the conclusion that the chemical constitution, the CO_2 production, and the latent period of growth are somehow positively correlated, and are to be distinguished from the phenomenon of growth and the factors which more directly determine it.
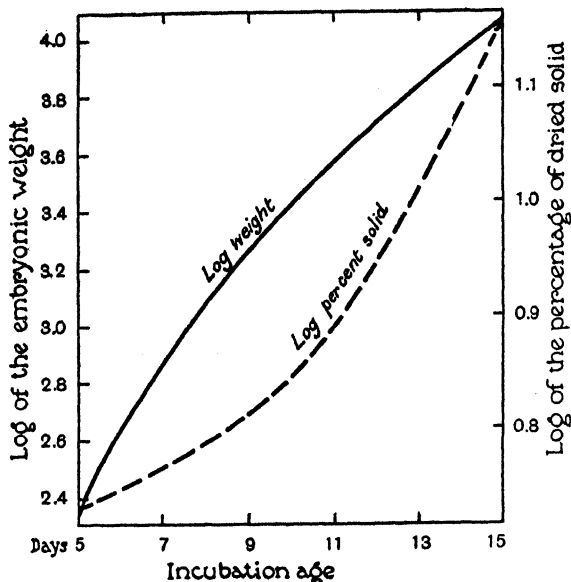
FIG. 7. The logarithm of the weight and the logarithm of the percentage of dried solids of chicken embryos as functions of time.

A Comparison of the Rates of Primary and Secondary Integration and Differentiation.

It has been pointed out that the general type curve to describe the changes in growth rate and form development (primary integration and differentiation) is quite different from that which describes the concentration of solids and the change in chemical form (secondary integration and differentiation). If observations were made at but two incubation ages it would appear that with time there was a progressive and concomitant decrease in growth and metabolic rates together with the concentration of solid substance and a differentia-

tion of outer and inner form. That this would be an inaccurate conclusion is evident.

If the period from the 5th to the 15th day is chosen and the curves for the logarithm of the embryonic weight and the logarithm of the per cent of dry substance are compared (Fig. 7) it will be evident that
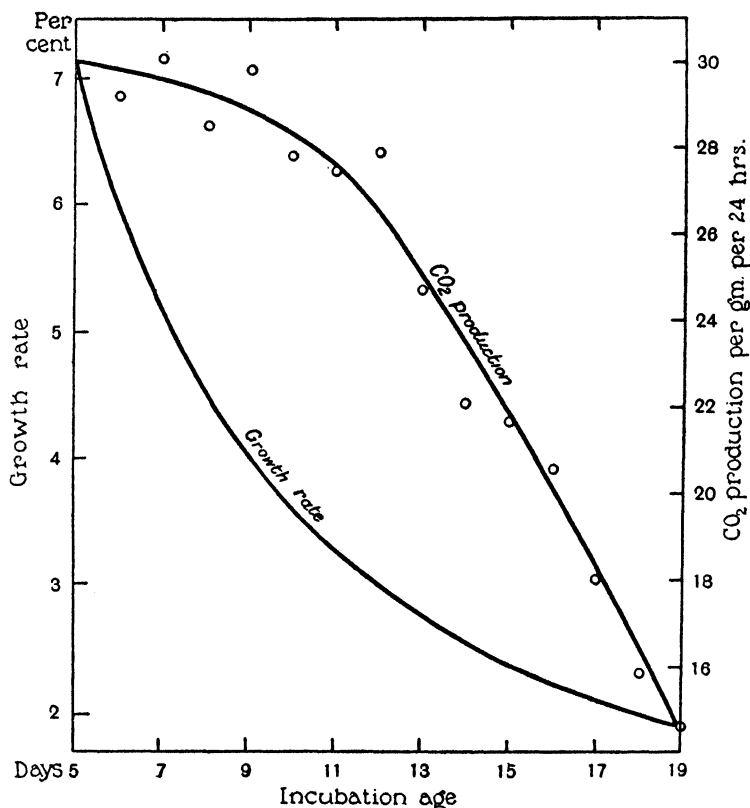
FIG. 8. The percentage growth rate and the rate of carbon dioxide production per gm. of embryo as functions of time.

the greatest relative increase in weight occurs in the beginning of the period under observation whereas in the differentiation of chemical form it occurs at the end. Or again, if growth rate is compared with metabolic rate, the same phenomenon is observed (Fig. 8). It would seem that in the beginning of this particular part of the cycle (5th

to the 19th day) the growth rate decreased markedly without much change either in metabolism or in chemical form; later, when the growth rate has reached relative constancy, internal chemical differentiation goes on apace and correlated with this is a decline in CO_2 production. It is as if the catabolic activity (or function) of the living protoplasm was the exciting cause or furnished conditions for certain chemical changes (differentiation of internal form) which in turn lead to a decrease in catabolic rate. If "aliveness" is measured by the velocity of chemical activity (heat production) an organism may in this sense be said to dig its own grave. The more abundant its manifestations of life the greater will be its rate of senescence.

DISCUSSION AND SUMMARY.

The chief results of the studies here reported have been (1) the correlation between growth as a whole and the differentiation of gross form (primary redistributions or simple evolution), and (2) the correlation between internal integration or concentration and the differentiation of chemical form (secondary redistributions or compound evolution). With the latter are also associated the catabolic rate and the latent period or reaction time after implantation in plasma as demonstrated in tissue culture experiments. Moreover, it has been shown that these two chief developmental processes occur at different rates, and that they undergo their greatest changes in rate at different periods of embryonic life. Corresponding with Robertson's growth acceleration periods there may be three cycles or rhythms of which the embryonic phase is the first, each composed of a period of growth followed by a period of differentiation. This conception is somewhat analogous to Roux's notion of dividing the life span into two chief periods (1) embryonic for the growth of organ rudiments and (2) post-embryonic, characterized by functional development. The first period of total growth and form differentiation seems to cover the time when the main, but bare, outline or scaffolding of the organism is laid down. The second period, correlated as it is with catabolism (function), corresponds, not in time but as a phenomenon, with Roux's period of functional form development.

To Sir Frederick G. Hopkins, F.R.S., for his encouragement and advice the thanks of the author are due.

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EXPLANATION OF PLATES.

PLATES 4 AND 5.

FIGS. 1, *a* and 1, *b*. Microscopic drawings of the chicken heart removed after 2.6, 2.7, 3.2, 3.9, 4.4, 5.8, 7.7, and 15.8 days of incubation respectively. The successive drawings are spaced so as to represent equal degrees of change in form differentiation. Thus the times taken to make the pictured transformations were 0.1, 0.5, 0.7, 0.5, 1.4, 1.9, and 8.1 days respectively. The reciprocals of these numbers have been used as indices of the velocity of gross form differentiation.
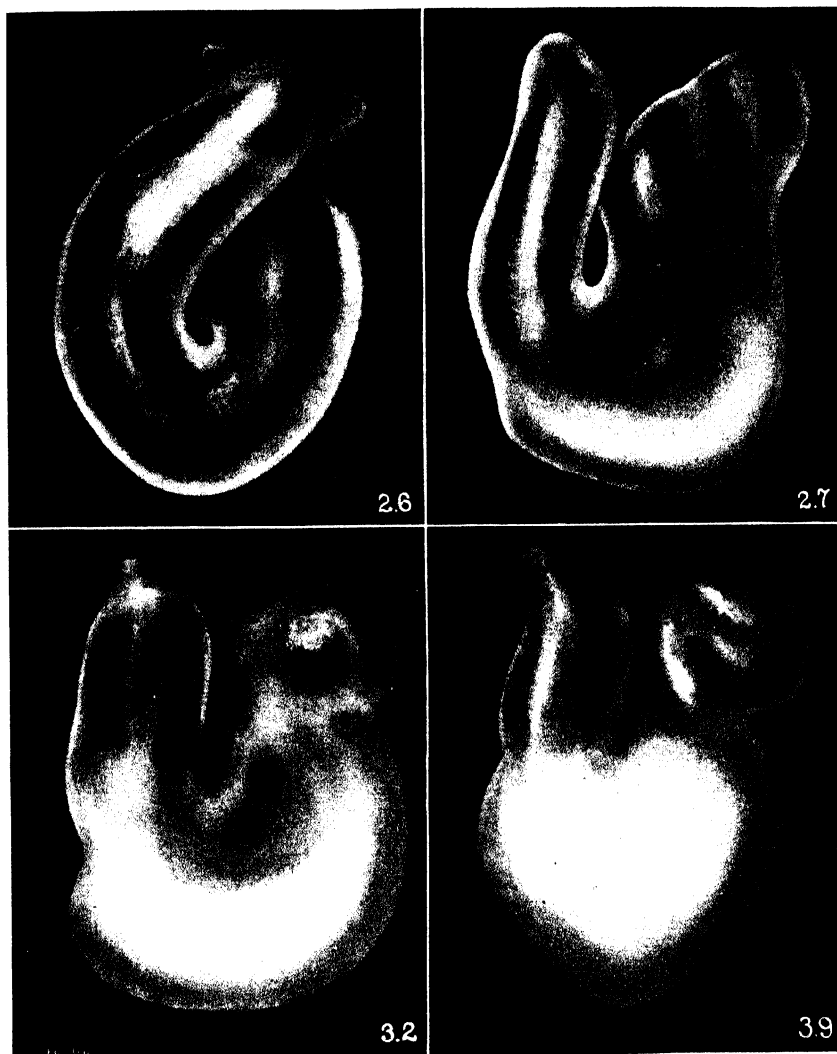
FIG. 1, *a*.
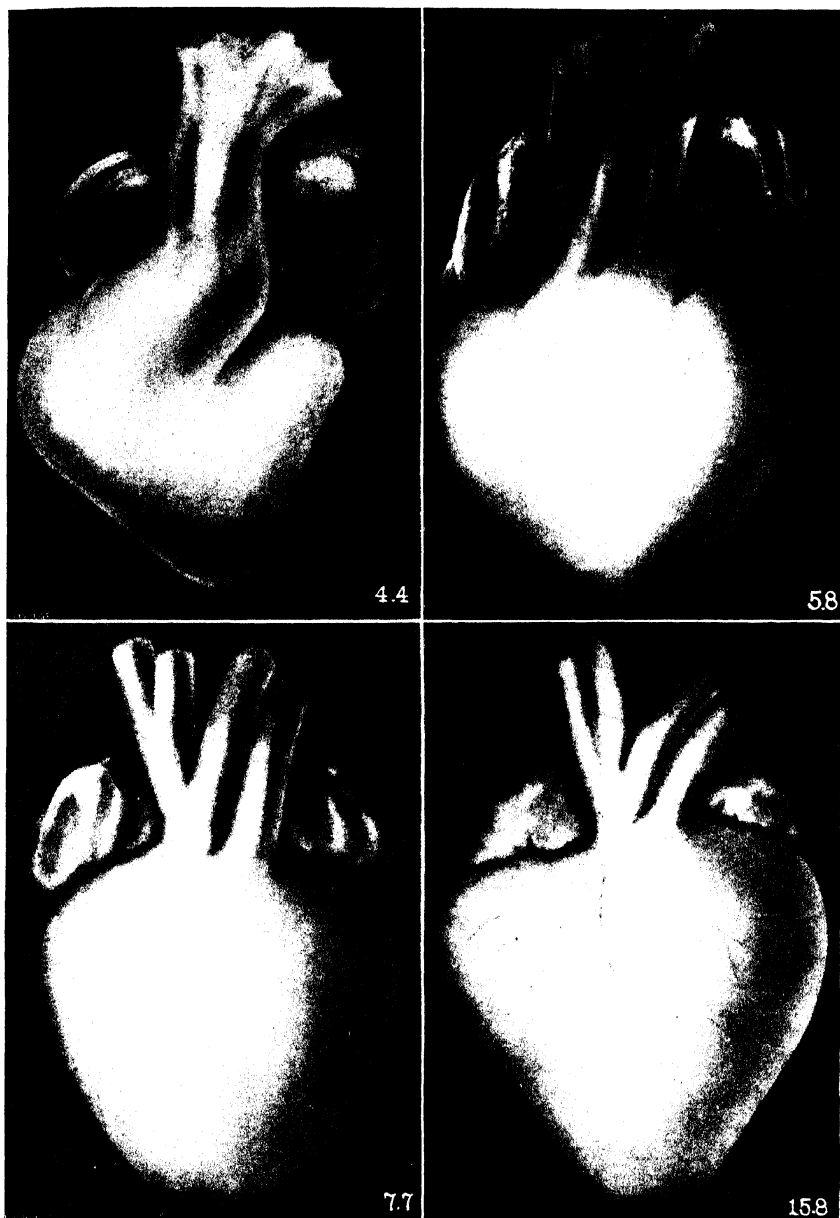
FIG. 1, *b*.

PHYSIOLOGICAL ONTOGENY.

A. Chicken Embryos.

IX. The Iodine Reaction for the Quantitative Determination of Glutathione in the Tissues As a Function of Age.

By HENRY A. MURRAY, Jr.

(From the Biochemical Laboratory, University of Cambridge, Cambridge, England.)

The importance of the sulphydryl compound, glutathione, as one of the thermostable components of the oxidation-reduction system of living cells has been established by Sir F. G. Hopkins (1) and his associates in the Biochemical Laboratory, Cambridge, and recently a method for the quantitative estimation of –SH groups in tissue has been proposed by Tunnicliffe (2). In a study of the chemical and physiological changes with age in chicken embryos, therefore, it would seem desirable to include an analysis of the tissues for glutathione. It would be interesting to know whether the concentration of this important substance is positively correlated with the velocity of such phenomena as growth and catabolism.

Method.

Tunnicliffe's technique was followed. Embryos of the same age were expeditiously separated from the rest of the egg contents and dropped into 10 cc. of a 10 per cent solution of trichloracetic acid. Enough eggs were opened to provide about 20 or 30 gm. of embryonic tissue. The embryos were immediately weighed, cut up, macerated in a mortar, and the homogeneous pulp filtered on a Büchner funnel and washed with 40 cc. of a 10 per cent solution of trichloracetic acid. Filtration was carried on with slight negative pressure. The filtrate was protein-free and clear. The extraction process was repeated three times and each filtrate titrated separately. ½ hour after the embryos were weighed the first filtrate was titrated. This was done with a 0.005 N solution of iodine using sodium nitroprusside as an outside indicator (1 cc. $N/200$ I_2 = 1.25 mg. reduced glutathione). It has been shown that glutathione in the tissues is practically all in the reduced form.

It is uncertain whether this is a test for glutathione alone or includes other sulphydryl compounds of similar properties. For the present, however, the most probable hypothesis is assumed to be true, namely that all the −SH groups present are those of glutathione.

RESULTS.

The analyses showed that the percentage of glutathione in the embryo as a whole decreased with age (Table I). Having previously determined the water content of the tissues it was possible to express

TABLE I.

Glutathione (−SH Group) Content of Chicken Embryos As a Function of Age·

| 1 | 2 | 3 | 4 | 5 | 6 | 7 | Le Breton and Schaeffer (4). | |
Incubation age.	No. of determinations.	Average No. of embryos for each determination.	Reduced glutathione in whole embryo.	Standard error. ±	Dry substance* in whole embryo.	Reduced glutathione in dry substance.	8 Age.	9 $\frac{NP \times 100}{NT - NP}$
days			*per cent*		*per cent*	*per cent*	*days*	
6.8	1	32	0.0221		5.80	0.38	8	10.70
11.8	5	5	0.0319	0.0033	8.47	0.38	9	9.54
12.7	2	5	0.0308	0.0001	9.71	0.32	11	7.60
13.7	4	3	0.0321	0.0027	11.70	0.27	13	7.08
14.7	2	4	0.0318	0.0010	14.00	0.23	14	6.09
15.7	2	3	0.0245	0.0033	15.86	0.15	15	5.40
16.7	7	2	0.0186	0.0010	16.97	0.11	16	4.19
17.7	2	2	0.0198	0.0023	17.55	0.11	17	4.40
18.7	6	2	0.0163	0.0012	17.70	0.09	18	3.90
19.7	3	1	0.0179	0.0018	17.70	0.10	19	3.78
							20	3.70

* The figures quoted in this column were obtained in a previous study (5).

the result in terms of dry weight (Fig. 1). In 2 weeks of incubation the concentration falls to a figure approximately one-fourth of its former value. This graph is not dissimilar to curves previously obtained for the changes with age in other chemical substances (type B curve) and therefore is correlated with catabolism rather than with anabolism (3). It resembles rather closely the curve for the percentage of chloride in dry tissue.

Le Breton and Schaeffer sought to obtain by chemical analysis the change with age in the nucleocytoplasmic ratio in chicken embryos.

They made determinations of purine nitrogen and total nitrogen, the former as an index of nuclear substance, and the difference between the two as an index of extranuclear material. Thus, the nucleocyto-plasmic ratio would be represented by the ratio $\dfrac{NP \times 100}{NT - NP}$. The values obtained by Le Breton and Schaeffer are included in the accom-
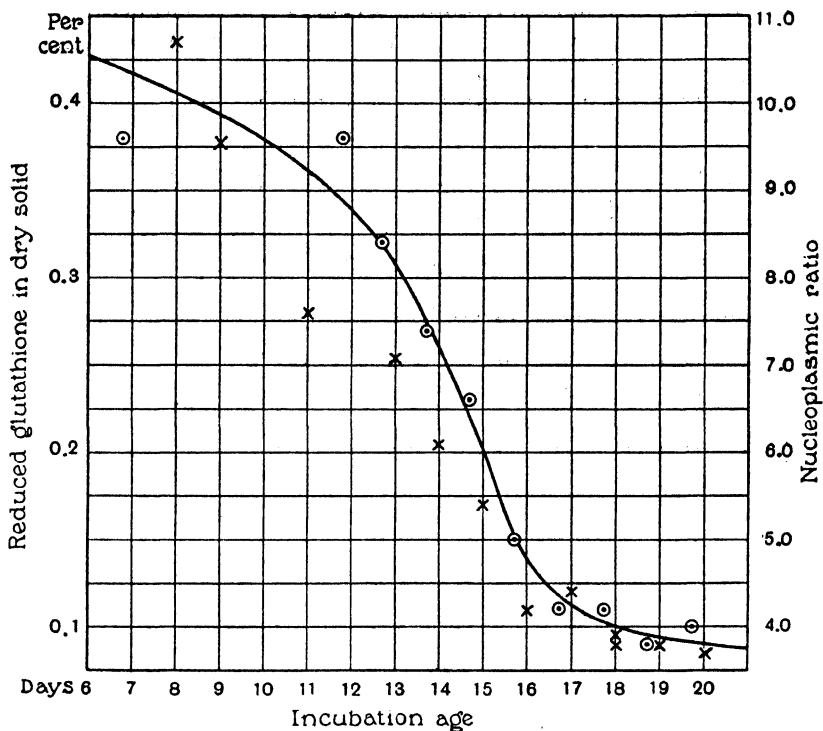
FIG. 1. The −SH (reduced glutathione expressed as percentage of dried solid) of chicken embryos (⊙) and Le Breton and Schaeffer's nucleoplasmic ratio (×) as functions of the incubation age of chicken embryos.

panying figure so that they may be compared with the changes in the percentage of glutathione in the dry substance.

All our curves have shown that there is little chemical differentia-tion between the 5th and 8th days of incubation, a fact which suggests that if points had been obtained by Le Breton and Schaeffer before

the 8th day of incubation, it would have been shown that the negative acceleration of the declining nucleocytoplasmic ratio was less in the early days of incubation than extrapolations from their published curves would seem to indicate.

SUMMARY.

The iodine reaction to determine —SH groups in tissues according to the technique of Tunnicliffe showed that the percentage concentration of such compounds in the dried substance of chicken embryos declines with age chiefly during the third quarter of the incubation period.

The author takes pleasure in acknowledging his indebtedness to Sir Frederick G. Hopkins, F. R. S.

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THE SHAPE OF THE MAMMALIAN ERYTHROCYTE AND ITS RESPIRATORY FUNCTION. II.*

BY ERIC PONDER.

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In a previous paper (1) it has been pointed out that a section through the poles of a mammalian red cell of typical biconcave form approximates to one of the equipotential curves of Cayley, and that, although such a curve does not fit the shape of the cell with any exactness, the approach of the cell surface to an equipotential surface is interesting in view of the respiratory function of the erythrocyte. The equipotential surfaces considered were obtained by revolving equipotential curves about their minor axis, a process which causes the two sinks associated with the curves to form a ring; this ring was then regarded as a circular sink to which the rotated curves were equipotential surfaces.

Now this procedure is not quite accurate, and gives only an approximation to the true result. The rotated curve, strictly speaking, does not form an equipotential surface to a ring, but a section through the poles of that surface gives a curve equipotential to the two points in which the ring is cut by the plane of section—a different thing, although the difference may not be an obvious one. The difference is not a great one, and in the paper referred to it was ignored, partly to avoid such complexity as would have obscured the general principles which were dealt with, and partly because the treatment was admittedly more suggestive than complete. However, it has been since suggested to us that, in view of the interest now attached to the question of diffusion gradients in the erythrocyte, a more complete study of these equipotential surfaces would be desirable; in this paper, therefore, we shall treat the matter in a more rigid manner.

Suppose that we wish to find the equipotential surfaces to a circular

source or sink of radius a. In Fig. 1, let $PB = r'$, the distance of a point P on the required surface, from a point on the ring. Let the distance from the centre of the ring, O, to P be r. Let the plane OAP be perpendicular to the plane of the ring, and let OA and OB make an angle φ, while OA and OP make an angle θ.
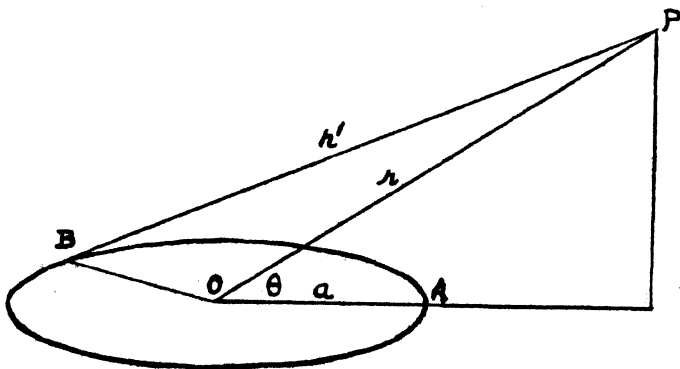
FIG. 1.

We first require PB in terms of φ and θ. By simple trigonometry we find that

$$PB = r^2 + a^2 - 2ar \cdot \cos \theta \cdot \cos \varphi$$

The surface, to be equipotential to the ring, must have

$$P = m \int \frac{ds}{r'}$$

or, since $ds = a \cdot d\varphi$,

$$P = 2m \int_0^\pi \frac{a \cdot d\varphi}{\sqrt{a^2 + r^2 - 2ar \cdot \cos \theta \cdot \cos \varphi}}$$

Here m is some constant, which we can ignore in the meantime. Continuing with the integral, and putting $\varphi = 2\psi$, $\psi = \dfrac{\pi}{2} - \chi$.

$$\frac{P}{2} = \int_0^{\pi/2} \frac{2a\, d\chi}{\sqrt{a^2 + r^2 - 2ar \cdot \cos \theta \cdot (2 \cdot \sin^2 \chi - 1)}}$$

$$= \frac{2a}{\sqrt{a^2 + r^2 + 2ar \cdot \cos \theta}} \int_0^{\pi/2} \frac{d\chi}{\sqrt{1 - k^2 \sin^2 \chi}},$$

where

$$k^2 = \frac{4ar \cdot \cos \theta}{a^2 + r^2 + 2ar \cdot \cos \theta} \tag{1}$$

This last integral is a well known form, being the first complete elliptic integral of the modulus k.

The necessary condition for the equipotential surface is, therefore, that

$$\frac{K_k}{\sqrt{a^2 + r^2 + 2ar \cdot \cos \theta}} = \text{a constant} = \psi \tag{2}$$

This is a very awkward expression to deal with, and admits of no further simplification. The best way to obtain the equipotential surfaces which it describes is to assign certain values to r and θ, and thus to arrive at the several surfaces in a semigraphical manner. We proceed as follows.

First we assign a convenient value to a, the diameter of the ring which is the circular source or sink—an easily worked value is 10 units. Next we proceed to introduce various values for r and θ into (1) and (2), and to evaluate the constant, ψ, in each case. To facilitate labour, we first of all take cases where $\theta = 0°$ and where $\theta = 90°$, and obtain the following values for ψ.

	$\theta = 0°$.	$\theta = 90°$.
$r = 1.0$	0.157	0.156
2.0	0.158	0.154
2.3	—	0.153
3.0	—	0.150
5.0	0.166	0.140
12.0	0.177	0.100
12.7	0.153	—
13.0	0.148	—
15.0	0.122	0.087

Now the value of r when $\theta = 90°$ gives us half the least thickness of the cell, and the value of r when $\theta = 0°$ gives the semidiameter. We know the ratio of these two measurements to be approximately $1:5.5$, and we have, therefore, to select from the values of ψ in the above table one which will give this ratio between r when $\theta = 90°$ and r when

$\theta = 0°$. The value 0.153 is such a one, for it gives values for r of 2.3 and 12.7 respectively. The surface $\psi = 0.153$ is therefore one which will fit the red cell as regards its two axes, and is accordingly to be worked out in full. We obtain the following values:—

$\theta =$	0°.	10°.	20°.	30°.	45°.	60°.	90°.
$r =$	12.7	12	10.6	8.0	4.5	3.0	2.3

The curve is shown in Fig. 2, in which the position of the ring, which is, of course, seen as two points in the section, is indicated by S in the quadrant shown. OS, therefore, is equal to a.
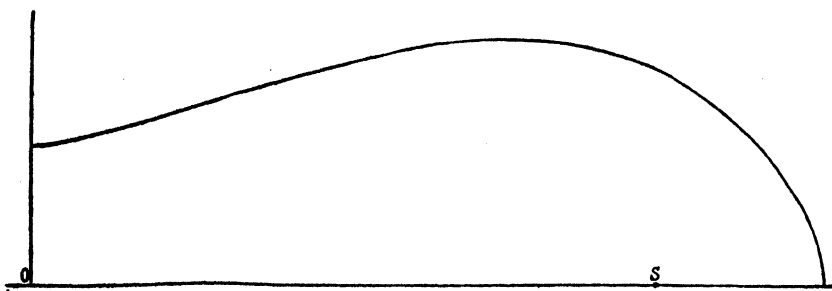
FIG. 2.

To this equipotential surface we may now apply the same tests as applied to the curves of Cayley, to see if it fits the form of the red cell. By construction, the ratio of the least thickness to the diameter is correct, being approximately 5.5. The ratio of the greatest thickness to the diameter is 3.1, and is rather large. The crucial test lies in the comparison of the volume enclosed by the surface with the volume of the cell, which is known with a fair degree of accuracy to be about $110\mu^3$. By the application of Pappus' theorem the volume enclosed by the equipotential surface works out as $144\mu^3$, if the major axis of the curve be reduced to a scale so that it equals 8.8, the mean diameter of the human erythrocyte. This volume is about 45 per cent too large. In the case of the curve of Cayley which provides the best fit for the cell (1), the volume was found to be $196\mu^3$; the equipotential surface here considered is therefore the better, but still does not fit the cell in more than an approximate way.

It will be observed that, since we have

$$\frac{P}{2a \cdot \pi \cdot m} = \psi$$

the effect of altering the value of a, the diameter of the circular sink, will be to alter the numerical value of the equipotential surface, but not to alter its form.

Allied to the curves of Cayley and to the curves which we now describe is the family of curves known as the Cassinian ovals. Certain of these present an appearance suggestive of that of the erythrocyte. Although these curves are of no interest in connection with diffusion gradients in the cell, we have investigated them to see whether any of them might be taken as fitting the form of the cell; the result may be briefly stated by saying that the best of them encloses too great a volume, and that they may accordingly be dismissed from notice.

The summary of our previous paper thus remains unchanged. The surface of the red cell is not exactly an equipotential one to any simple source or sink, but it approximates to an equipotential surface, a fact which is of interest and importance in connection with its respiratory function.

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A LATENT PERIOD IN THE ACTION OF COPPER ON RESPIRATION.

BY S. F. COOK.

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I.

A preceding paper (Cook, 1925-26) has discussed the general effect of salts of copper and of other metals on the respiration of *Aspergillus niger*. It was demonstrated that the toxic action followed certain definite laws and could be formulated in a mathematical system. Mention was made of a peculiarity in the case of copper, wherein this element differed from the others studied. When copper chloride acts on *Aspergillus niger* there is at first no change in the rate of respiration, but later the production of carbon dioxide decreases in the manner characteristic of the action of all the heavy metals studied. The time which elapses between the introduction of the copper and the beginning of the drop in respiration rate has been termed the *latent period*.

Save where otherwise stated, the methods used in investigating this latent period were the same as those used in studying the other toxic effects of copper, mercury, and silver. All work on respiration was done by the indicator method with the Osterhout respiration machine as previously described. Fig. 1 shows a typical curve for the effect of copper chloride, including latent period and subsequent drop.

It might appear that the figure does not consist of a straight line followed by a distinct and clearly differentiated drop, but that it is really a continuous curve starting with the introduction of the copper, falling imperceptibly for a while and then with increasing velocity. In this case the curve should show a sharp bend toward the end of

the latent period, and not a cusp as here represented. That this is not true is maintained for two reasons:

1. Several hundred experiments were performed with different concentrations of copper and particular attention was paid to the readings at or near the end of the latent period. But in no instance was there any indication of a rounded curve at that point. The last reading of the latent period was always the same as the preceding readings, of which there were several, and there was no indication of
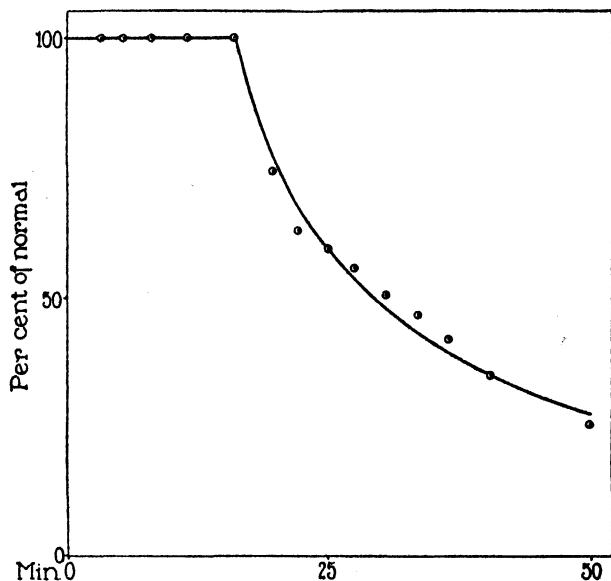
FIG. 1. Curve with copper chloride 0.25 M. Typical single experiment.

a gradual falling off in respiration during that time. The first reading after the latent period, particularly with high concentrations, was usually a considerable distance below the normal level, so far indeed as to leave no doubt that the curve should be drawn as in Fig. 1.

2. If the curve is continuous from the zero abscissa then it must all represent the effect of the copper on the respiratory system. This system has been represented as a series of consecutive reactions $A \rightarrow X \rightarrow Y \rightarrow Z$ which is altered by the copper through its effect upon the velocity constants. If the entire curve represents the effect of

the copper on respiration then the entire curve should be reproducible by means of the formula for two consecutive reactions. A curve of this sort cannot be so duplicated (see Figs. 2 and 3). It seems reasonable to suppose, then, that the latent period is due to some delay whereby the copper does not directly affect the respiratory system until after a definite interval of time.
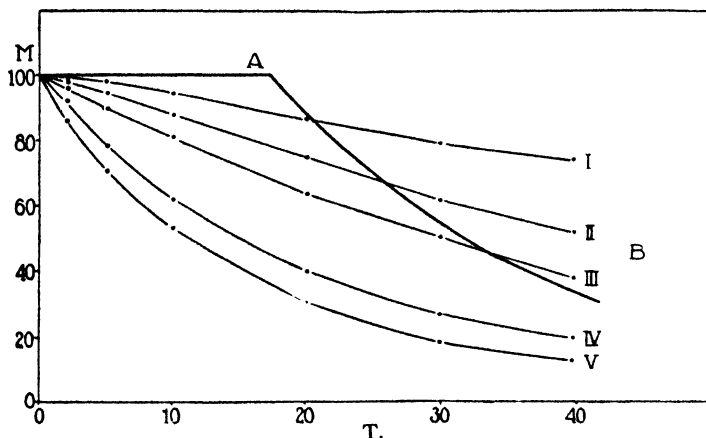
FIG. 2. Solid line is curve with copper chloride 0.02 M $K_v = 0.021$. Broken lines are curves calculated by the formula for consecutive reactions:

$$M = Ae^{-K_3 t} + B \left(\frac{K_1}{K_2 + K_1} \right) \left(e^{-K_1 t} - e^{-K_3 t} \right)$$

where $A = 1$, $B = 1$, $K_1 = 0.03$, and in

Curve I $K_2 = 0.1$
" II $K_2 = 0.08$
" III $K_2 = 0.05$
" IV $K_2 = 0.04$
" V $K_2 = 0.031$

II.

The question arises whether the mechanism responsible for the delay is of a chemical or physical nature, and whether it takes place outside or inside the cell. If the temperature is raised, say from 25° to 35°, it is found that the latent period is shortened, and on plotting the temperature against the latent period Fig. 4 is ob-
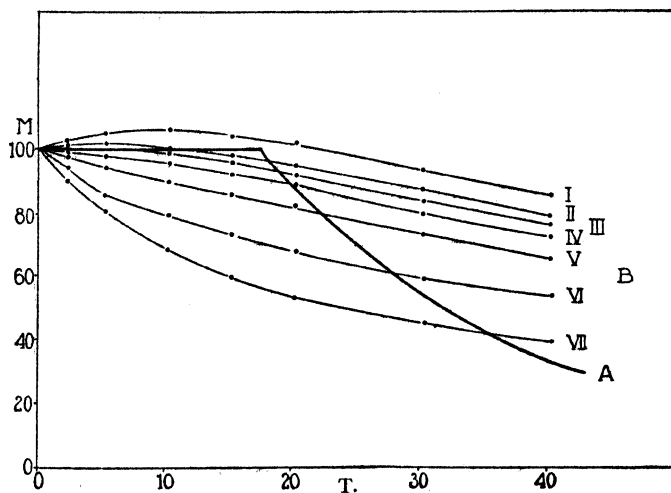
FIG. 3. Solid line same as Fig. 2.
Broken lines are curves calculated as in Fig. 2 where $A = 1$, $K_1 = 0.01$, $K_2 = 0.1$, and in

Curve I	B	= 11.818
" II	B	= 10.909
" III	B	= 10.454
" IV	B	= 10.000
" V	B	= 9.090
" VI	B	= 7.272
" VII	B	= 5.454
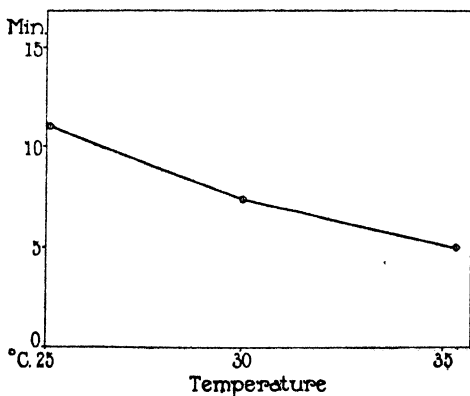
FIG. 4. The temperature plotted against the length of the latent period.

tained. This shows that the temperature coefficient is nearly 2, a number characteristic of chemical rather than of physical processes. Of course the criterion is not absolute, for some physical processes have a coefficient as high as 2; but it may be considered fairly strong evidence in favor of the intervention of a chemical reaction rather than of a physical hold-up. Only three points on the temperature scale were used and further analysis using the recently developed method of Crozier (1924–25) might yield more information, but the general character of the system may be ascertained from only a few points.

Further evidence is found in the fact that if the toxic solution is removed from the respiration chamber (for the method see the preceding paper) before the end of the latent period, the drop in respiration will take place as usual and the rate of production of carbon dioxide will fall to a new, lower level and there remain (see Fig. 6). This phenomenon will be discussed again, but it may be pointed out here that it is very difficult to conceive of a trapping mechanism, such as an impermeable cell wall or membrane, or the adsorption of a substance on a surface, which would give this effect. If the copper is prevented from reaching the oxidative mechanism of the cell by any physical obstruction or barrier, which must first be saturated or broken down, it is hard to see how the copper can eventually get through, after it has been removed from the outside, and injure the cell. In the case of an ordinary physical process dependent on the presence of the external solution, that process could scarcely continue after the removal of the external solution. It is possible, however, if there is no physical barrier, and the copper gets into the cell immediately, that there might be a chemical system whereby the copper which had already penetrated might remain and ultimately damage if not kill the cell.

If the latent period is nothing more than the time required for a chemical reaction to take place somewhere inside the cell wall, then it ought to be possible to demonstrate the presence of copper inside the cell before the end of the latent period. For this purpose there are two methods of procedure, (a) the direct method, to test chemically for the presence of copper in the cell, and (b) an indirect method, to see if copper can injure the cell within the requisite time using criteria other than that of respiration.

A. *Aspergillus* does not lend itself readily to microchemical analysis because of the very small size of the cells. Therefore, an undetermined species of the stonewort *Nitella* was used. The material came from Woods Hole, Massachusetts, and was characterized by very large cells (1 to 4 inches long) and watery sap. *Nitella* gave a good respiration curve with copper. It was a little more sensitive than *Aspergillus* but the latent period was longer, being about 20 minutes with 0.01 M copper chloride. After determining the respiration curve, cells were put in the same concentration of copper for varying lengths of time and the sap then squeezed out (Osterhout, 1921–22; Irwin, 1922–23). 1 or 2 cc. of sap were usually obtained and tested with potassium ethyl xanthate. This reagent gives a yellow to brown color with copper, depending on the concentration, and is sensitive to about 0.00001 molar. In several experiments no color was obtained till 20 to 25 minutes exposure to 0.01 M copper chloride. Thereafter the color steadily increased. The result might be taken to signify that copper was not in the cell till after 20 minutes, that is till after the latent period. It is more probable that it was there but in such minute quantities as to be below the threshold for determination with xanthate.

Analogous experiments were performed with *Valonia sp.*, a marine green alga growing in shoal waters at Bermuda. Unfortunately this organism did not produce sufficient carbon dioxide to measure in the respiration machine and consequently it is not known whether it has a latent period or not. In any case copper penetrates it rapidly. In 0.01 M copper sulfate in sea water a good xanthate test could be obtained after 15 minutes exposure. By evaporating 50 cc. of the sap nearly to dryness a good test was obtained after 5 minutes exposure, indicating the presence of copper in the cell at that time. If there is a latent period of respiration it is probably longer than 5 minutes, at this concentration of copper. Thus the direct method is suggestive but not conclusive. If the latent period with *Valonia* were certainly known to be more than 5 minutes it would be conclusive.

B. An indirect method was applied to the same organisms, *Nitella* and *Valonia*. With *Nitella* separate lots of twenty-five cells each were exposed for periods of varying duration to 0.01 M copper chlo-

ride. They were then placed in water and allowed to stand several hours before testing. A control lot of the same number of cells, which were not exposed to copper, was placed in water with the injured. The criterion of injury was the turgidity of the cells. If the cell was able to support its own weight when held by one end it was considered uninjured. If it broke and fell it was considered injured if not dead. This criterion makes use of a purely arbitrary point in the process of injury, for the loss of turgidity is progressive, but it is very convenient and useful. The results showed that even a very short exposure to copper (2 minutes) caused some of the cells to lose their stiffness. Of those which had been exposed for longer periods more were injured, until in the lots exposed for an hour or longer all were injured (or killed). In the control lot none, whatever, had lost its normal turgidity. This shows quite clearly that the copper injured the cell in many cases before 2 minutes had elapsed. In order to injure it must have penetrated at least as far as the plasma membrane and must have been exerting a toxic effect on the protoplasm even while it was not affecting the respiration.

Similar results were obtained with *Valonia*. In this instance the criterion of injury was the presence of sulfate ion in the sap. *Valonia* normally does not give a test for SO_4 (Wodehouse, 1917), but if injured will allow this ion to enter. The same rough correspondence was observed between the duration of exposure to copper and the percentage of cells injured; *i.e.*, giving the sulfate test. In fact it was here quite clearly logarithmic. The shortest exposure to copper showing injury to any of the cells was 10 seconds, in which experiment several of the cells were injured. This indicates that with *Valonia* at least copper penetrates from the start, regardless of any effect it may have on the respiration. Taken in conjunction with very similar results on *Nitella* where the latent period is known, it furnishes good evidence that copper is not held up on the outside by a physical barrier but that the latent period corresponds to a process which occurs inside the cell. We may say then, for present purposes at least, that the latent period is a chemical trapping mechanism situated inside the cell.

It should be noted here that when, with *Aspergillus*, the concentration is varied the duration of the latent period varies likewise

(see Fig. 10). The relation between concentration and latent period is interesting, for it is the inverse of that observed in the case of velocity of toxic action; *i.e.*, the length of the latent period varies inversely as a constant power of the concentration. Thus log latent period $= \dfrac{1}{B} \log C + \log A$, or latent period $= A \, C^{-B}$. This fact also points to a chemical rather than to a physical explanation of the latent period and will be further discussed.

<center>III.</center>

It should now be possible to construct a hypothetical system, on a chemical basis, whereby the process underlying the latent period may be illustrated if not fully explained. It would be impossible at the present stage to state exactly what are the reactions involved. But a system which duplicates the experimental results should at least serve to bring out the characteristics of these reactions, even though it could not tell us the precise nature of the substances concerned. Any explanation must account for the following four experimental results.

(1) that a latent period exists;

(2) that on removing the external solution the latent period continues and that subsequently the rate of carbon dioxide production comes to an equilibrium below the normal level;

(3) that the length of the latent period varies inversely as a constant power of the external concentration of copper; and

(4) that there is a latent period with some metals (copper, iron, and tin) and none with others (mercury, silver, hydrogen, etc.).

1. It has been suggested previously that the normal oxidations of the cell take place in a series of catenary reactions each one of which is controlled by a catalyst and is characterized by its own velocity constant. The toxic effect of any metal is held to be due to the alteration of these velocity constants. But the metal in its raw state, so to speak, cannot do this. It must previously be activated, or undergo some chemical change (*e.g.* combination, change of valence, etc.). This activation has been assumed to take place through a reversible reaction involving a combination with a cell constituent called T. Thus if M represents the metal we can write

$M + T \rightleftharpoons MT$. To account for the latent period we must extend this hypothesis. Before combining with T let us say that M must diffuse from the external solution into the cell and that when it gets inside it exists in the form MR. This does not imply that MR is a compound between M and some cell constituent R, but is simply used for convenience to distinguish between the metal outside and inside the cell. The metal may undergo some change of state as from the ionic to the molecular form, from a hydrated to a non-hydrated condition or *vice versa*. Its exact nature is immaterial. We may treat it as if the metal simply diffused into the cell, and call the external metal M and the internal metal MR. We then have this system:

$$MR + T \underset{K_4}{\overset{K_3}{\rightleftharpoons}} MRT \quad \text{or} \quad CuR + T \underset{K_4}{\overset{K_3}{\rightleftharpoons}} CuRT$$
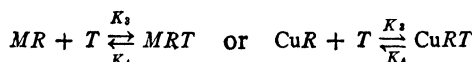
The next step is to analyze this system with respect to the rate of formation of $CuRT$. In calculations concerning these reactions it is permissible to let the velocity constants be whatever seems desirable. We may also assume that the rate of penetration of Cu into the cell is much more rapid than the rate of its diffusion out again. This might be due to the physical nature of the cell wall or membrane or to chemical reactions involving the copper as it entered or left the protoplasm. Indeed the temperature coefficient (approximately 2) indicates that a chemical reaction might possibly be connected with the entrance of the copper into the cell. Furthermore we may say that the rate of entrance of the copper is greater than the rate of combination of the copper with T. If the difference between these rates is great enough the penetration of the copper will be practically instantaneous (a few seconds) while the formation of $CuRT$ will take a considerably longer time (a matter of minutes). For practical purposes we may consider that the concentration of the copper inside the cell (CuR) will be at equilibrium with that of the copper outside (Cu) before the reaction $CuR + T \rightleftharpoons CuRT$ begins. The simplest assumption is that the concentrations are the same outside and inside or at least that the ratio between them is always the same.

On this basis the reaction $CuR + T \underset{K_4}{\overset{K_3}{\rightleftharpoons}} CuRT$ may be considered

by itself. Starting with CuR at an arbitrary concentration of, say, 100, and considering the reaction as monomolecular on the ground that T is present in great excess, it is possible to plot the formation of CuRT.

If x is the amount of CuRT formed after time, t, a is the concentration of CuR at the start and ξ is the amount of CuRT present at equilibrium, then

$$\frac{dx}{dt} = K_3 (a - \xi) - K_4 \xi.$$

But at equilibrium $\dfrac{dx}{dt}$ is zero, and $K_3 (a - \xi) - K_4 \xi = 0.$

Then

$$K_4 = \frac{K_3 (a - \xi)}{\xi}.$$

But at any moment during the reaction,

$$\frac{dx}{dt} = K_3 (a - x) - K_4 x;$$

or, substituting,

$$\frac{dx}{dt} = K_3 (a - x) - K_3 \frac{a - \xi}{\xi} x.$$

This equation when integrated takes the form

$$\frac{1}{t} \log \frac{\xi}{\xi - x} = \frac{K_3 a}{\xi}.$$

But, since

$$K_4 = \frac{K_3 (a - \xi)}{\xi}, \xi = \frac{K_3 a}{K_3 + K_4}.$$

Substituting and rearranging,

$$\log \frac{\xi}{\xi - x} = (K_3 + K_4) t$$

Expressed in the exponential form:

$$x = \xi - \xi e^{-(K_3 + K_4)}$$

and, finally, substituting for ξ, we get

$$x = \frac{K_3\, a \left(1 - e^{-(K_3 + K_4)\,t}\right)}{K_3 + K_4}$$

If we let a have the value of 100, where $K_3 = 0.016$ and $K_4 = 0.001$, and plot the curve of the formation of $CuRT$ we get Fig. 5, Curve A. It will be observed that the rate is rapid at first and progressively becomes slower and reaches equilibrium only at infinity. Since the toxic action of copper depends on its presence in the form $CuRT$
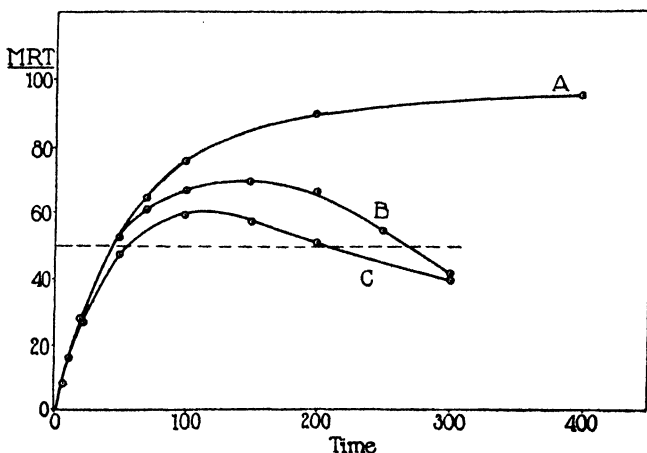
Fig. 5. Curve A shows the amount of activated copper ($CuRT$), or in the case of any metal (MRT), plotted against time. For formula see text. Curves B and C show the effect of removing the toxic agent after 50 and 10 units of time respectively. For method see text.

it is obvious that there can be no toxic action without $CuRT$. The hypothesis here advanced to account for the existence of the latent period is this: a certain definite amount of $CuRT$ must be formed before the action on the respiratory system can begin. Since a measurable time must elapse before such a quantity is formed there will be a period of inactivity previous to the observed fall in respiration, and this is called the latent period. There is involved the idea of a threshold, analogous to, although differing in many respects from, other thresholds known to biology and chemistry. The latent

period here observed may be compared on the one hand to the threshold of nerve stimulus and on the other to certain induction periods observed by chemists. It resembles the former in that a definite intensity must be attained before there is any visible response on the part of the organism, but here the stimulus is chemical whereas with nerve it is more likely physical (*cf.* Lillie, 1923). The resemblance to the chemical induction periods is based on the fact that in both cases there is a chemical reaction taking place for a considerable time before there are any apparent effects (*cf.* Forbes, Estill, and Walker, 1922). Hecht (1923-24 etc.) has made similar assumptions with regard to the effect of light on *Mya*. There seems to be no reason for not maintaining, in the case of copper, that there is a threshold which must be passed in order for the toxic activity to occur, and that the threshold is a function of the formation of activated copper, $CuRT$. In Fig. 5 we may place the threshold arbitrarily at 50 units of $CuRT$, or at a concentration of 50. On the scale here used about 30 units of time are consumed during the latent period. If we say 30 minutes we shall not be far from the experimental facts.

The effect of the other metals may be considered at this point. It was suggested previously that they are all activated in a manner similar to that assumed for copper. If so there should be a latent period with these elements likewise. It is the belief of the writer that there is one, but that it is often too short to be detected experimentally. The longest latent period observed with copper was 120 minutes. If that with mercury, for equivalent concentration, were one hundredth as long it would be about 1 minute, and a latent period of 1 minute would be impossible to observe when the subsequent drop is very slow. With higher concentrations there would be still less chance of detection. It is only necessary to assume, with mercury for instance, that the value of the product $HgRK_3$ is much larger than with copper. Thus $HgRT$ is formed much more rapidly and the threshold is passed immediately. If we let the presence or absence of a latent period depend on the relative value of K_3 then it follows logically that one of the effects of all metals is a latent period of greater or less duration, only a few of which are long enough to permit experimental detection. Iron and tin, as well as copper, show noticeable latent periods but they are only about half

as long. We can say that FeRT and SnRT are formed about twice as fast as CuRT but very much slower than HgRT and AgRT. The metals, therefore, may be distinguished by the rates at which they are activated.

2. Reference has been made to phenomena which occur when the toxic substance is removed shortly after its introduction. Figs. 6 and 7 show the effect of the removal of copper after 3 and 17 minutes exposure. In the first instance the latent period continued a little longer than usual, then the respiration rate fell off and gradually
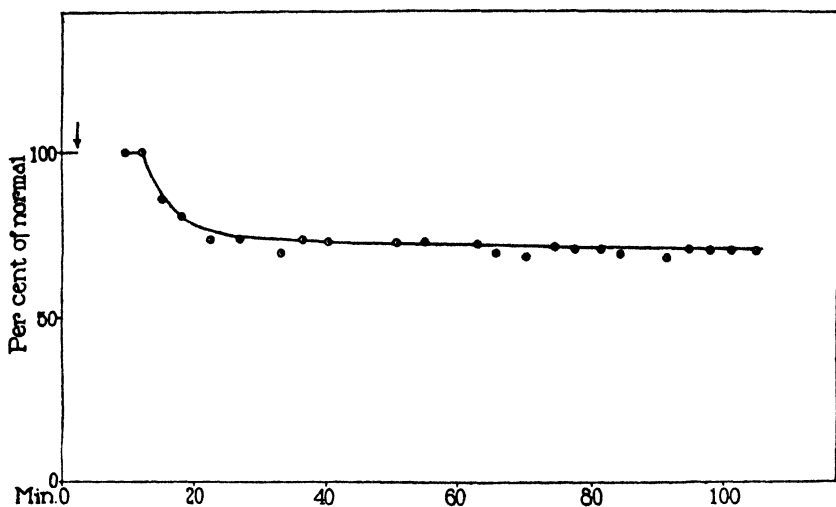
FIG. 6. The effect of removing copper chloride 0.0075 M after 3 minutes exposure.

attained a new level at about 70 per cent of the normal. In the second example the removal of the toxic agent occurred after the end of the latent period and the respiration continued to fall, gradually reaching equilibrium at about 25 per cent of the normal. Whether any secondary, permanent injury was sustained which ultimately killed the organism was not ascertained, but the equilibrium level was held for several hours at least and secondary effects do not enter into the present discussion.

Fig. 8 illustrates the result of the removal of mercury. After removal the equilibrium was attained before the next reading could

be taken, and was held during the time of the experiment.   This is in direct contrast to copper where the equilibrium was only gradu-
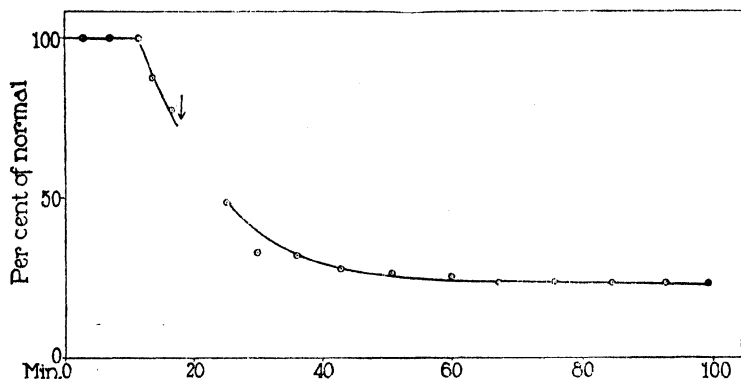
FIG. 7. The effect of removing copper chloride 0.0075 M after 17 minutes exposure.
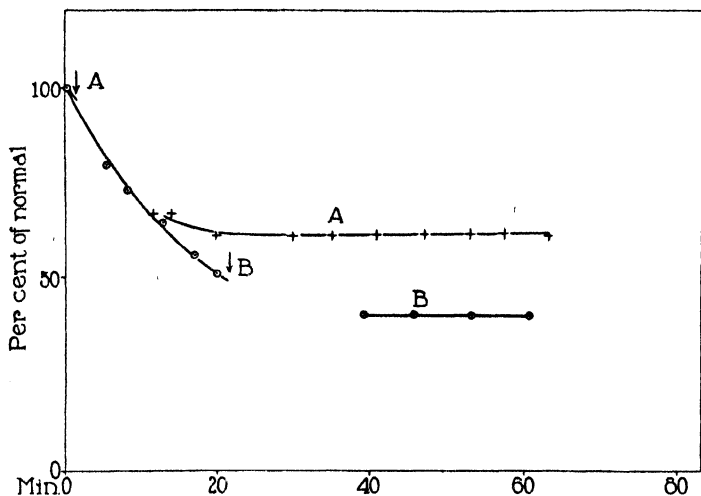
FIG. 8. The effect of removing mercuric chloride 0.0002 M after 2 and 20 minutes exposure.

ally attained.   Fig. 9 shows the effect of the removal of silver, (A) at the peak of the curve and (B) on its descending limb.   The same

situation obtains here also, for equilibrium is attained before read-
ings are resumed and, curiously, bears no reference to the rise in
respiration, characteristic of the action of silver. These results can
be interpreted in the light of the previously outlined hypothesis.

During the formation of $CuRT$ (to consider the case of copper)
the concentration of CuR was held constant by being in equilibrium
with the copper outside the cell, and in the formula

$$x = \frac{K_3\, a\, \left(1 - e^{-(K_3 + K_4)}\right)}{K_3 + K_4}$$
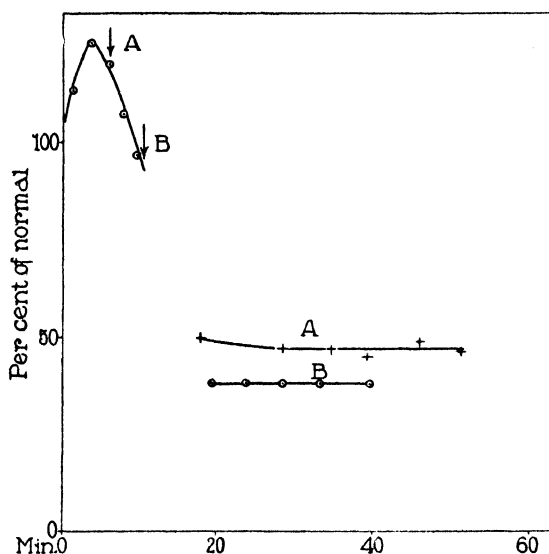
FIG. 9. The effect of removing silver nitrate 0.0002 M after 5 and 10 minutes
exposure.

a was kept at the arbitrary concentration of 100. Now if the out-
side copper (Cu) is removed so that its concentration is practically
zero then the situation is as follows: Any uncombined copper in the
cell (CuR) escapes into the outside fluid and is for our purposes lost.
There will be no further diffusion of the copper into the cell, but on
the other hand there will be a slow diffusion outward, whereby CuR
in the cell will become lost as Cu in the surrounding medium. At the
same time the reaction $CuR + T \rightleftharpoons CuRT$ is still going on but some

of the copper is continually being lost. This loss, or outward diffusion, may be assumed to go on at a rate always proportional to the amount of copper (CuR) left in the cell. It will thus follow the law of an irreversible, monomolecular reaction and will have a velocity constant K_2. Stated another way the amount of CuR remaining in the cell will equal $ae^{-K_2 t}$ where a is the amount of CuR present when the outside copper was removed. This situation may be represented thus: $\downarrow \text{Cu} \xleftarrow{K_2} \text{Cu}R + T \underset{K_4}{\overset{K_3}{\rightleftharpoons}} \text{Cu}RT$. If, as we have supposed in the case of copper K_2 is smaller than K_3 more copper will for a while be converted into CuRT than will escape from the cell. If it is larger the reverse will be true, although CuRT will increase for a relatively short time, depending on K_4. For, suppose the product CuRTK_4 is larger than the product CuRK_3, then the reaction Cu$R \rightleftharpoons$ CuRT will run to the left and CuRT will diminish from the moment of the removal of the outside solution. If the reverse is true then CuRT will increase until CuR is nearly exhausted, and the rate of exhaustion will depend on the size of K_2. In the case of copper the constants assumed were $K_2 = 0.003$, $K_3 = 0.016$, and $K_4 = 0.001$.

In the formula a was constant at 100, but now the outward diffusion of CuR must be considered. If CuR is being lost at the rate K_2 then the value of $ae^{-K_2 t}$ can be substituted for a and the equation may be written:

$$ x = \frac{K_3 a e^{-K_2 t} \left(1 - e^{-(K_3 + K_4)t}\right)}{K_3 + K_4}. $$

Curves B and C in Fig. 5 were calculated by means of this formula. It is evident that the value of x (CuRT) rises for a while and then falls. If we take 50 as the threshold we see that after removing the reagent the CuRT increases to the threshold, but takes longer in the process, exerts a toxic action for a short time, and then falls again. During the period that CuRT is above the threshold the respiration falls, and when CuRT drops below the threshold the oxidation system regains its equilibrium but at a lower level. This lower level indicates that the respiratory catalysts have been permanently reduced by the removal of A and X. The duration of the toxic activ-

ity is dependent on the exposure to the copper solution and consequently on the quantity of CuRT formed before removing the copper.

When mercury is removed something similar takes place. The velocity constants of this reaction have been assumed to be greater than in the reaction involving copper, and the further assumption may be made that here K_4 is greater than K_3. Then HgRT decreases from the moment the external mercury is removed HgR diffuses and since all the reaction velocities are much greater HgRT very rapidly sinks below the threshold. So rapid is the decomposition of HgRT that when the next readings are taken the toxic action has ceased and the respiratory system $A \rightarrow X \rightarrow Y \rightarrow Z$ has had time to come to equilibrium in accordance with the now reduced quantities of A and X.

The same reasoning applies to the case of silver. Here the striking feature is that if the silver is removed at the peak of the curve, the new level is far below the normal. The curve of silver has been explained by saying that in the reactions $A \rightarrow X \rightarrow Y \rightarrow Z$ the velocity constant of $X \rightarrow Y$ is increased more than that of $Y \rightarrow Z$. The reaction $A \rightarrow X$ ceases while the metal is present, and A is being continually reduced in quantity. This results in an increase in Y for a short time and in consequent acceleration of the carbon dioxide production. Meanwhile X is being exhausted and ultimately the value of Y falls. If the silver is removed and the toxic action stopped while Y is at a maximum the normal velocity constants will be restored very soon. Y will fall to a new value in accordance with the now permanently reduced value of A and X. This must all take place in the interval between the readings.

To summarize it may be stated that the phenomena observed on removing the toxic agent may be accounted for by differences produced in the velocity constants governing the reactions $M + R \rightleftharpoons MR + T \rightleftharpoons MRT$.

3. There remains the effect of the concentration on the length of the latent period. The experimental data (see Fig. 10) indicate that there is an inverse logarithmic relation between them; i.e., latent period $= A \ C^{-B}$ in which C is concentration and A and B are constants. The complete reaction for the activation of copper implies

molecular proportions of a high order.   In discussing the effect of the concentration on the toxic action in general, use was made in a previous paper of the equilibrium constant of the second half of the reaction and the experimental curve was duplicated.   It was necessary to assume the molecular proportions mentioned in order to account for the fractional exponent in the equation $K_{\bullet} = A\ C^{B}$. For present purposes we will disregard the fractional exponent and assume that the reaction $CuR + T \rightleftharpoons CuRT$ involves only the simple proportions of 1:1.   If we were to attempt to duplicate the *slope* of the curve of latent period and concentration it would be necessary to assume the former complex proportions, but for simplicity of
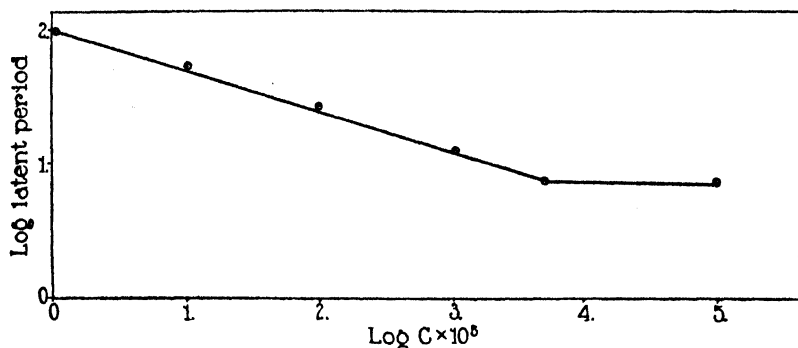
FIG. 10. The logarithm of the latent period (*L.P.*) plotted against the logarithm of the concentration (copper chloride).

calculation the reaction $10(CuR)_5 + 2T_{25} \rightleftharpoons 25(CuRT)_2$ may be replaced by: $CuR + T \rightleftharpoons CuRT$.

For concentrations within the experimental range we may regard T as greatly in excess of CuR and treat it as a reversible monomolecular reaction, for a bimolecular reaction may be so treated when one of its components is in excess.   In calculating Fig. 5 the external concentration, and therefore that of CuR (a in the formula) was called 100.   If T is indefinitely large with respect to a then we may take different values for a and determine t, the length of the latent period.   This has been done in Fig. 11 for values of a between 60 and 10,000, when x is held constant at 40, and the logarithm of a plotted against the logarithm of t.   It is evident that $\log t = \dfrac{1}{B}$

log a, or $t = A \ C^{-B}$. B equals unity because of the molecular pro-
portions assumed in the equation. If the proportions were correct
B would be a fraction as in the experimental case. It is clear, how-
ever, that the relation between the length of the latent period and the
concentration is logarithmic, both in the experiments and in the
theoretical system.

As a approaches the value of T, which is finite (however large),
the relations change. When the values of a and T are nearly equal
the system is clearly bimolecular and the monomolecular formula
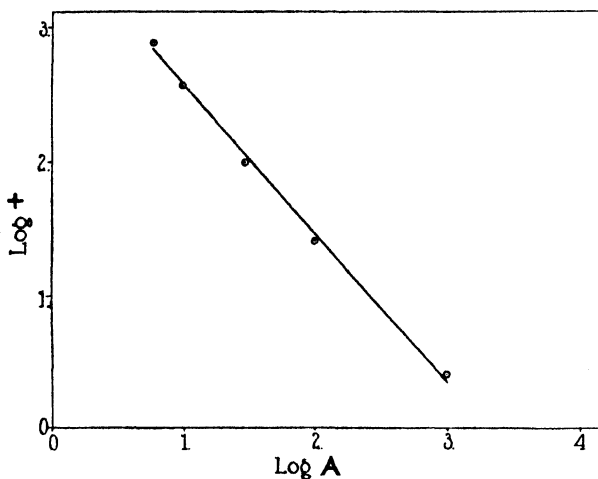
FIG. 11. The logarithm of the time plotted against the logarithm of A. For
method see text.

cannot be used. It is evident that as a becomes greater than T
the similarity to a monomolecular reaction will again appear, but now
with a as the component in excess. Variation in its concentration
will have little effect because the value of CuRT is approaching the
value of T beyond which it cannot go. The result will be, then, that
the logarithmic relation will hold until high concentrations of the
toxic reagent are reached, at which time the sloping line in the plot
will become more nearly horizontal. That this is true experimentally
is shown in Fig. 10.

SUMMARY.

1. When copper chloride is allowed to act on *Aspergillus niger* there is at first a period during which there is no change in the rate of the production of carbon dioxide, following which the rate of respiration falls. The interval of no change is called the latent period.

2. When the copper is removed from the external solution before the end of the latent period this interval is prolonged. The rate of respiration then falls to a new level below the normal level.

3. Experiments on *Nitella* and on *Valonia* indicate that the copper penetrates the cell almost immediately.

4. The length of the latent period varies inversely as a constant power of the concentration of the copper.

5. These results are explained by assuming that the copper is made active in the respiration system by means of a reversible reaction. By using appropriate velocity constants the experimental curves can be duplicated by calculated curves.

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STUDIES ON ENZYME ACTION.

XXXV. Lipase Actions of Extracts of Tissues of Rabbits at Different Ages.

By HELEN MILLER NOYES, K. GEORGE FALK, and EMIL J. BAUMANN.

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INTRODUCTION.

In a study[1] of the lipase actions on ten different esters, of aqueous extracts of whole rats whose ages ranged from 3 days before birth to 3 years 15 days, it was found that the "pictures" of the relative actions on the different substrates changed progressively with increasing age of the rat. For the embryo and the youngest rats, the curves approached those given by the Flexner-Jobling rat carcinoma and by a number of tumors of human origin, changing to a type characteristic of the adult rat, and appearing to revert again to some extent toward the embryonic type for the oldest rats. The absolute actions on the different esters were found to increase and then to decrease again as the rats became older, the extent of the changes differing with the various esters.[2]

As it was desired to study the changes in these enzyme actions of individual tissues with increase in age of the animal, and as the young rat is too small to use for this purpose, at the suggestion of Dr. C. B. Davenport the rabbit was chosen for this investigation. A study of a

[1] Falk, K. G., Noyes, H. M., and Sugiura, K., *J. Gen. Physiol.*, 1925–26, viii, 75.

[2] Some early studies showed that the lipase activity of rabbit, pig, and sheep embryos increased with age (Buxton, B. H., and Shaffer, P., *J. Med. Research*, 1904–05, xiii, 549), and that the actions on ethyl butyrate of the liver and intestines of the adult pig were greater than those of the embryo (Mendel, L. B., and Leavenworth, C. S., *Am. J. Physiol.*, 1908, xxi, 95).

number of tissues of the adult rabbit[3] indicated differences in enzyme relations which should be of value in the study of similar properties with rabbits of different ages.

In this paper, results will be presented for the lipase actions of the following tissues of the rabbit: lung, liver, kidney, leg muscle, heart muscle, skin, spleen, stomach, small intestine, and brain. The ages of the rabbits ranged from 8 days before birth to 5 years 11 months 2 days.

Experimental Methods.

Rabbits of various breeds were used. Several of them were obtained from the Carnegie Institution, Station for Experimental Evolution, Cold Spring Harbor, while the six oldest were obtained from the Bussey Institution, Harvard University. For these the authors desire to thank Dr. C. B. Davenport and Dr. W. E. Castle. The ages of those which were obtained before birth were calculated on the assumption of a gestation period of 30 days. The very young rabbits were killed with ether, the older by a blow below the atlas. The tissues were removed immediately, passed through a meat chopper, or ground with sand in a mortar, taken up with water, toluene added, extracted at room temperature overnight, filtered through paper, cloudy liquids being obtained as a rule, and brought to pH 7.0. The conditions of testing the lipase actions were the same as those described previously; 15 cc. of solution, 3.4 milli-equivalents of each of the ten esters, 22 hours incubation at 37–38°, titration with 0.1 normal sodium hydroxide solution with phenolphthalein as indicator, duplicate and blank determinations, toluene present throughout.[4]

Experimental Results.

The experimental results will be given only for the tissues of rabbits whose ages are definitely known. As in previous papers, two methods of comparing the enzyme actions present themselves; either as relative

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[3] Noyes, H. M., and Falk, K. G., *J. Biol. Chem.*, 1924–25, lxii, 687.

[4] *Cf.* methods used in previous papers; Falk, K. G., Noyes, H. M., and Sugiura, K., *J. Biol. Chem.*, 1924, lix, 183, 213, 225; 1924–25, lxii, 697; Noyes, H. M., Sugiura, K., and Falk, K. G., *J. Cancer Research*, 1925, ix, 105; Sugiura, K., Noyes, H. M., and Falk, K. G., *J. Cancer Research*, 1925, ix, 129 and foot-notes 1 and 2.
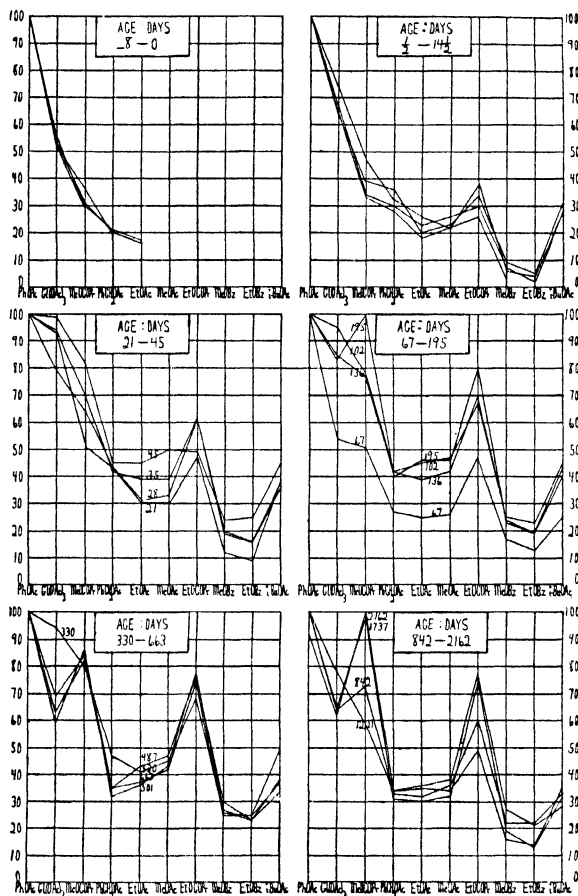
FIG. 1. *Kidney Extracts.* The relative actions given in the chart show a regular progression with increase in age of the rabbits. For the youngest, the type is very much like that found with the whole rat embryo, with the Flexner-Jobling rat carcinoma, and with a number of human tumors. As a matter of convenience, it may for the present be referred to as the embryonic type. A change in the type apparently begins at the age of 14 days and may be said to be complete at 195 days. The type then remains fairly constant except that with the oldest rabbits, a reversion toward the original younger type is appearing with the acetates. It is unnecessary to go into details with the individual esters, as the changes are very apparent. Some irregularities appear, at times with individual esters, very occasionally with a group of esters with one rabbit. Such irregularities are unavoidable, and in fact to be expected, in dealing with experimental data from separate biological units.

actions of a given extract on a number of esters, or as absolute actions. The results for any one tissue will be given in the form of curves for the relative actions and tables for the absolute actions in as much detail as seems advisable in each case to bring out the various relations.

TABLE I.

Hydrolyzing Actions in Tenths of Milli-Equivalents of Acid Produced by Kidney Extracts of Rabbits of Different Ages on the Indicated Esters.

Age.	PhOAc	Gl(OAc)₃	MeOCOPr	PhCH₂OAc	EtOAc	MeOAc	EtOCOPr	MeOBz	EtOBz	i-BuOAc
days										
−8 (dil.).	1.16	0.60	0.34							
−7 (dil.).	1.02	0.53	0.37	0.19						
−3 (dil.).	1.27	0.67	0.39	0.25	0.20					
0 (dil.).	1.69	0.93	0.51	0.36	0.28					
½ (dil.).	1.52	0.98	0.50	0.42	0.28	0.34				
2½	1.80	1.20	0.62	0.54	0.42	0.46	0.54	0.12	0.04	0.50
6½	1.74	1.11	0.67	0.62	0.34	0.42	0.59	·0.16	0.08	0.54
14½	1.68	1.25	0.79	0.54	0.43	0.37	0.64	0.10	0.06	0.47
21	2.35	2.22	1.19	1.04	0.72	0.72	1.10	0.28	0.21	0.89
28	2.58	2.03	1.65	1.10	0.79	0.86	1.58	0.51	0.42	0.94
35	3.60	3.38	2.53	1.51	1.42	1.40	2.18	0.69	0.59	1.36
45	3.75	3.72	3.07	1.69	1.70	1.88	1.82	0.90	0.94	1.70
67	4.01	2.17	2.04	1.08	0.99	1.05	1.87	0.67	0.54	1.02
102	4.19	3.99	3.24	1.78	1.86	1.96	2.82	1.07	0.97	1.85
136	3.75	3.20	2.89	1.56	1.45	1.58	2.57	0.89	0.70	1.51
195	4.88	4.05	4.90	1.98	2.23	2.23	3.90	1.13	0.93	2.13
330	4.19	3.95	3.36	1.99	1.73	1.89	2.86	1.09	1.03	2.07
487	3.44	2.16	2.81	1.20	1.44	1.56	2.62	1.04	0.80	1.32
501	3.14	1.86	2.70	0.99	1.14	1.36	2.43	0.85	0.73	1.07
663	2.73	1.89	2.30	0.95	1.00	1.14	2.01	0.67	0.67	1.01
842	3.79	2.44	2.75	1.25	1.22	1.36	2.29	0.84	0.82	1.26
1221	6.23	4.86	3.66	2.13	2.16	2.14	3.05	0.99	0.89	2.21
1737	3.31	2.16	3.24	1.04	0.99	1.05	2.44	0.64	0.42	1.06
2162	3.22	2.16	3.51	1.19	1.26	1.35	2.72	0.94	0.74	0.97

In the curves for showing the relative actions the esters are arranged along the abscissa axis in the same order as in the previous papers. As a rule, four curves are given on each plot; in some cases which will be indicated, three, five, or six are shown.

In the discussion of the results, the different physiological periods (epochs) of the life of the rabbit may be recalled; *i.e.*, embryonic, nursing and weaning, young, adult. The embryonic is quite uniformly 30 days. The nursing rabbit begins to eat food other than milk at about the age of 2 weeks, and, as a rule, is weaned at about 6 weeks. The onset of puberty is quite variable. In general, rabbits become sexually mature between 5 and 7 months of age.

The consideration of the absolute actions on the different esters, of the kidney extracts of the rabbits of different ages is essential for a satisfactory understanding of the changes in these enzyme actions. These values are given in Table I.

The results for the five youngest rabbits were obtained with extracts corresponding to 8.9 mg. original tissue per cc. final solution tested, the remaining results refer to double this concentration. The striking fact for these absolute actions is their large increase with increasing age, up to a certain point. For the older rabbits definite decreasing actions are observed with benzyl, ethyl, methyl, isobutyl acetates, methyl and ethyl benzoates; possible decreases with phenyl acetate and glyceryl triacetate, but no decreases with methyl and ethyl butyrates. The absolute actions for the 1221 days old rabbit are considerably larger than those for the rabbits somewhat younger and somewhat older. The relative actions, however, fall in line with those of the other rabbits. The absolute actions found with the other tissues of this rabbit are also greater than would be expected from the results with the other rabbits, but this apparently exceptional behavior is not reflected in the types or pictures of the relative actions which correspond to the results obtained with the other rabbits. These results can therefore only be recorded and no explanation suggested for them at present. It is also apparent that the very marked increases in the enzyme actions begin after the age of 14 days.

The physiological behavior of the rabbit kidney may be considered briefly in this connection. The embryonic kidney practically does not function. While the rabbit is on a milk diet only (up to the age of 10 days or so) the kidney activity is at a comparatively low level. After that the milk is supplemented by other foods and the kidney functions more actively. It is interesting that the change in enzyme action (both relative and absolute) becomes striking at this period. There
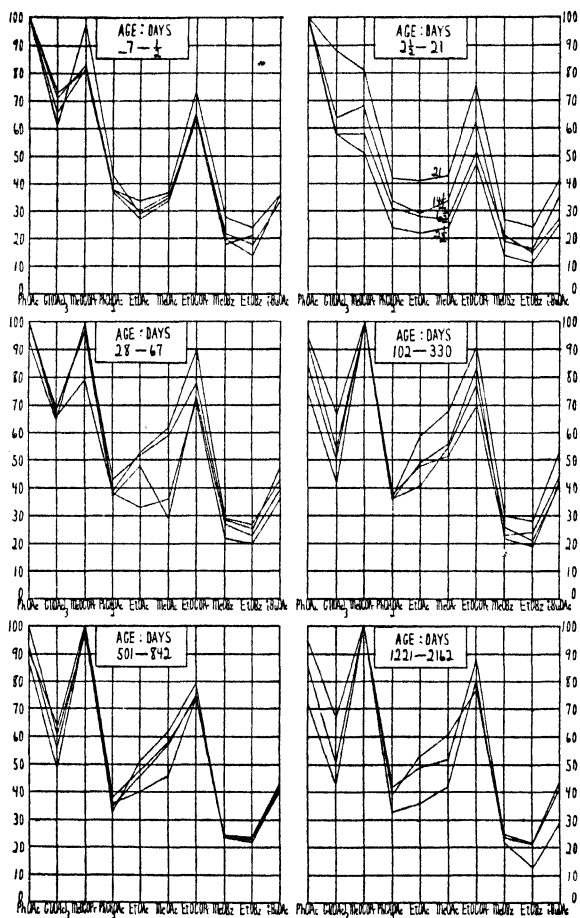
FIG. 2. *Liver Extracts.* These pictures or types of actions show considerable similarity throughout the life cycle of the rabbit. At one period, however, there is a definite difference. Between the ages of 2½ and 28 days there is a partial reversion in the direction of the embryonic type. The relative actions of the butyrates are markedly less here. Between the ages of −7 and 35 days, the actions on benzyl, ethyl and methyl acetates are much the same, thereafter the values increase in the order listed. The adult type may be said to be present after the age of 45 days. For the oldest rabbits, there are indications of a reversion as shown by the decrease in the three acetate ratios mentioned and by the decrease in the actions on the benzoates.

is also a possible correlation of physiological activity with the decrease in absolute lipase actions (small, but apparent) noted after puberty (about 200 days). The metabolism per kilogram is highest in the young. It decreases gradually until puberty when the curve flattens out and the rate of decrease with increasing age is very slow. The

TABLE II.

Hydrolyzing Actions in Tenths of Milli-Equivalents of Acid Produced by Liver Extracts of Rabbits of Different Ages on the Indicated Esters.

Age.	PhOAc	Gl(OAc)₃	MeOCOPr	PhCH₂OAc	EtOAc	MeOAc	EtOCOPr	MeOBz	EtOBz	i-BuOAc
days										
−7	2.90	2.06	2.40	1.11	0.99	1.09	2.12	0.81	0.71	1.05
−3	3.39	2.07	3.30	1.44	0.99	1.18	2.20	0.76	0.62	1.15
0	2.61	1.90	2.12	1.00	0.78	0.94	1.64	0.46	0.56	
½	2.83	1.86	2.32	1.06	0.77	0.96	1.79	0.57	0.41	1.01
2½	3.51	2.05	1.80	0.85	0.76	0.83	1.66	0.48	0.39	0.86
6½	3.01	1.74	1.74	0.92	0.83	0.81	1.54	0.62	0.46	0.82
14½	2.83	1.82	1.93	0.96	0.83	0.95	1.75	0.55	0.45	1.00
21	3.67	3.26	3.00	1.58	1.51	1.61	2.74	0.99	0.88	1.52
28	2.93	1.91	2.46	1.11	0.98	1.05	2.06	0.61	0.57	1.06
35	4.55	3.07	4.37	1.66	2.12	1.33	3.34	1.21	1.06	1.79
45	4.67	3.30	5.04	1.95	2.69	3.14	4.55	1.47	1.38	2.19
67	6.24	4.33	6.04	2.71	3.27	3.68	4.87	1.74	1.54	2.93
102	4.35	3.09	4.63	1.77	2.21	2.42	3.22	1.06	1.08	2.01
136	4.00	2.43	4.75	1.72	1.95	2.59	3.67	1.24	1.01	1.93
195	5.90	3.50	6.38	2.27	3.14	3.58	5.27	1.39	1.24	2.69
330	4.34	2.44	5.77	2.16	3.42	3.91	5.25	1.73	1.59	3.07
487	4.98	3.49	5.45	2.06	2.63	3.17	4.04	1.38	1.33	2.36
501	5.50	3.35	5.31	1.93	2.55	3.13	4.14	1.31	1.23	2.23
663	4.19	2.35	4.79	1.56	2.46	2.96	3.77	1.14	1.08	1.90
842	3.61	2.17	3.90	1.40	1.55	1.80	2.88	0.95	0.88	1.64
1221	6.18	4.36	6.51	2.53	3.48	3.96	5.01	1.66	1.40	2.84
1737	3.52	2.09	4.88	1.60	1.74	2.03	3.92	1.08	0.61	1.42
2162	5.20	3.16	6.14	2.55	3.01	3.19	5.38	1.49	1.29	2.49

metabolism is then lower per kilogram or per square meter of surface, but the total amount of substances excreted by the kidneys would probably not be much, if any, less.

The absolute actions of these liver extracts are shown in Table II. The results refer to the content of 17.8 mg. original solid per cc. of final solution tested.
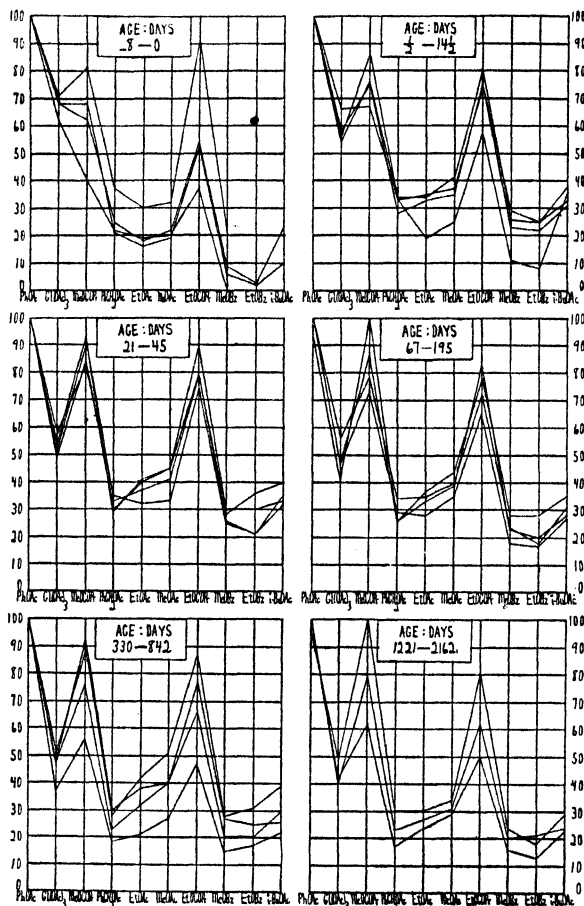
FIG. 3. *Lung Extracts.* The types of action for the three youngest rabbits approach the embryonic type. At birth or immediately thereafter, the type changes, the change being fairly complete at 14 to 21 days. In reaching this adult type, the actions on all the esters relative to phenyl acetate increase except that on glyceryl triacetate which decreases, and that on isobutyl acetate which does not change. For the oldest rabbits a certain amount of reversion is apparent. This can be seen in following the complete life cycle with the butyrates and with the benzoates which increase and finally decrease again. On the other hand the ratios of the actions on the butyrates to those on glyceryl triacetate increase from the embryo to the adult and remain constant and for the actions on benzyl acetate to those on ethyl benzoate (its isomer) decrease and then remain constant. Additional regularities involving various pairs of esters might be indicated but will not be given because of limitation of space.

In general terms these actions increase with increasing age of the rabbits. During the nursing period before the milk is supplemented by other foods, the first 2 weeks of life, the butyrates show a marked drop. All the esters show increases beginning at about 28 to 35 days,

TABLE III.

Hydrolyzing Actions in Tenths of Milli-Equivalents of Acid Produced by Liver Extracts of Rabbits of Different Ages on the Indicated Esters.

Age.	PhOAc	Gl(OAc)₃	MeOCOPr	PhCH₂OAc	EtOAc	MeOAc	EtOCOPr	MeOBz	EtOBz	i-BuOAc
days										
−8 (dil.).	0.81	0.50	0.32	0.18	0.15	0.16	0.30	0.00		
−7 (dil.).	0.65	0.44	0.40	0.16	0.12	0.14	0.35	0.06	0.02	0.15
−3	1.15	0.78	0.78	0.24	0.18	0.22	0.62	0.07	0.02	0.12
0 (dil.).	1.08	0.78	0.87	0.41	0.32	0.35	0.98	0.24		
½ (dil.).	1.29	0.85	0.87	0.42	0.24	0.32	0.74	0.14	0.10	0.46
2½	2.32	1.34	1.74	0.66	0.76	0.82	1.74	0.54	0.50	0.72
6½	2.45	1.39	1.86	0.80	0.86	0.90	1.80	0.72	0.62	0.80
14½	2.43	1.36	2.08	0.82	0.82	1.00	1.94	0.64	0.60	0.92
21	2.85	1.38	2.39	0.86	1.12	1.28	2.51	0.86	0.84	0.93
28	2.57	1.49	2.10	0.89	0.82	0.86	1.90	0.64	0.54	0.82
35	3.19	1.72	2.96	0.94	1.32	1.44	2.51	0.84	0.68	1.12
45	2.40	1.22	2.15	0.80	0.88	0.98	1.90	0.68	0.86	0.96
67	3.68	2.05	2.87	1.24	1.30	1.47	2.64	0.90	0.68	1.14
102	2.70	1.26	2.31	0.70	0.99	1.19	2.11	0.76	0.75	0.95
136	3.50	1.64	2.59	1.00	0.97	1.23	2.26	0.64	0.60	0.96
195	3.38	1.49	3.65	0.95	1.22	1.44	3.04	0.82	0.72	1.04
330	2.39	1.23	2.10	0.68	1.01	1.22	2.08	0.68	0.74	0.94
501	4.34	2.02	3.30	1.02	1.41	1.70	2.85	0.88	0.87	1.29
663	3.69	1.38	2.07	0.66	0.79	0.98	1.75	0.57	0.61	0.81
842	2.44	1.16	2.25	0.72	0.92	0.97	1.88	0.67	0.60	0.64
1221	4.53	1.89	2.79	0.78	1.09	1.34	2.28	0.73	0.61	1.01
1737	3.31	1.71	3.49	1.06	1.05	1.20	2.77	0.85	0.64	1.00
2162	4.41	1.78	3.49	1.03	1.20	1.38	2.74	0.88	0.94	1.08

these changes after weaning are especially large with the normal alkyl esters, methyl and ethyl butyrates and acetates.

With reference to the physiological changes, it may be stated that the liver may, and probably does, function during late embryonic life. During the nursing period the diet is restricted to an ideal food. There is then less "detoxication" by the liver—formation of conjugate
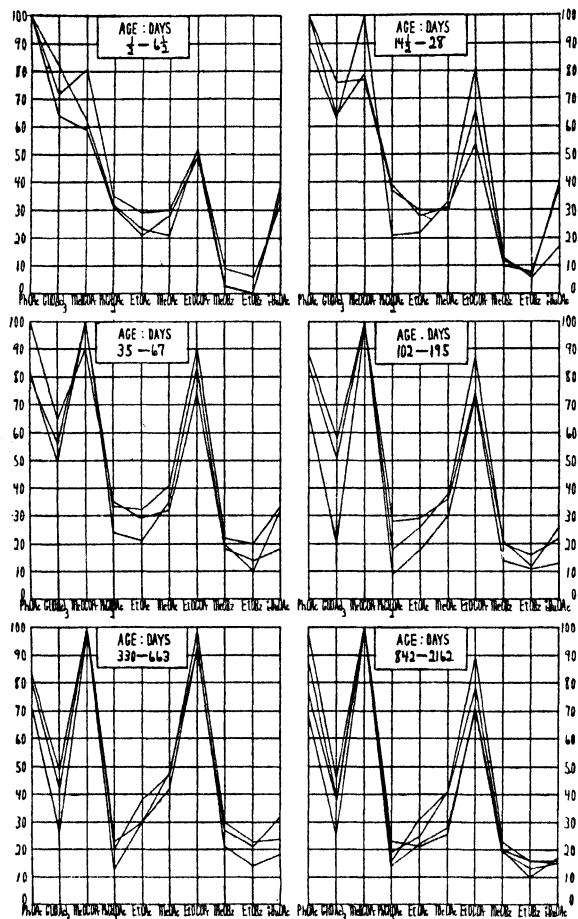
FIG. 4. *Skin Extracts.* Curves for three ages are shown in the first five plots, four on the sixth. No skins of embryo rabbits were studied. Comparing the types of the skins· of adult rabbits, which are markedly constant, with those of the younger rabbits, a difference is seen in that the results for the younger approach to a certain extent the embryo type of enzyme action, and which consequently suggest that with skin of embryos, the results would show completely the embryonic picture for the enzyme actions.   This is brought out in the increasing relative actions of the butyrates and the benzoates.   The actions on benzyl, ethyl, and methyl acetates,—much the same for the younger rabbits, increasing in the stated order of the esters for the older,—are of interest.

acids such as glycuronic acid, etc.—in which the liver is supposed to play a part. This may account for the reversion observed in the enzyme actions, both relative and absolute, during the nursing period.

The absolute actions of the lung extracts are shown in Table III. The lung extracts of the rabbits aged -8, -7, 0, and $\frac{1}{2}$ days refer to concentrations of 8.9 mg. tissue per cc. of solution tested, the remaining extracts were made up with 17.8 mg. per cc.

TABLE IV.

Hydrolyzing Actions in Tenths of Milli-Equivalents of Acid Produced by Skin Extracts of Rabbits of Different Ages on the Indicated Esters.

Age.	PhOAc	Gl(OAc)₃	MeOCOPr	PhCH₂OAc	EtOAc	MeOAc	EtOCOPr	MeOBz	EtOBz	i-BuOAc
days										
$\frac{1}{2}$	0.66	0.54	0.41	0.21	0.15	0.14	0.34	0.02	0.00	0.25
$2\frac{1}{2}$	0.71	0.45	0.42	0.22	0.15	0.20	0.34	0.02	0.00	0.26
$6\frac{1}{2}$	0.69	0.50	0.56	0.24	0.20	0.21	0.35	0.06	0.04	0.22
$14\frac{1}{2}$	0.80	0.51	0.63	0.31	0.22	0.25	0.43	0.08	0.06	0.30
21	0.56	0.40	0.63	0.13	0.14	0.21	0.51	0.08	0.04	0.11
28	0.83	0.63	0.63	0.31	0.25	0.24	0.53	0.10	0.06	0.33
35	0.59	0.36	0.72	0.17	0.15	0.25	0.60	0.13	0.10	0.13
45	0.72	0.50	0.90	0.30	0.29	0.37	0.81	0.20	0.18	0.30
67	0.82	0.53	0.74	0.29	0.24	0.26	0.61	0.16	0.08	0.26
102	0.84	0.51	1.00	0.18	0.26	0.38	0.87	0.20	0.16	0.22
136	0.89	0.59	1.01	0.28	0.29	0.36	0.75	0.21	0.12	0.26
195	0.91	0.28	1.35	0.12	0.24	0.41	0.98	0.19	0.15	0.18
330	0.70	0.37	0.88	0.18	0.33	0.41	0.86	0.26	0.20	0.21
501	0.70	0.27	0.99	0.13	0.30	0.42	0.91	0.21	0.14	0.18
663	0.64	0.38	0.77	0.18	0.23	0.37	0.71	0.21	0.16	0.25
842	0.61	0.30	0.80	0.15	0.20	0.33	0.62	0.16	0.13	0.12
1221	0.58	0.28	0.61	0.10	0.19	0.25	0.54	0.14	0.10	0.10
1737	0.85	0.37	0.98	0.23	0.21	0.25	0.70	0.20	0.10	0.17
2162	0.73	0.28	1.08	0.15	0.24	0.31	0.76	0.20	0.14	0.16

There is evidently an increase in the absolute enzyme action at or in the close neighborhood of birth. The increases on the various esters continue up to apparent maxima whose exact points or times are difficult to determine because of the irregularities in the individual cases but which appear to occur at about 14 days. No real decreases in the absolute actions can be stated to occur with the oldest rabbits
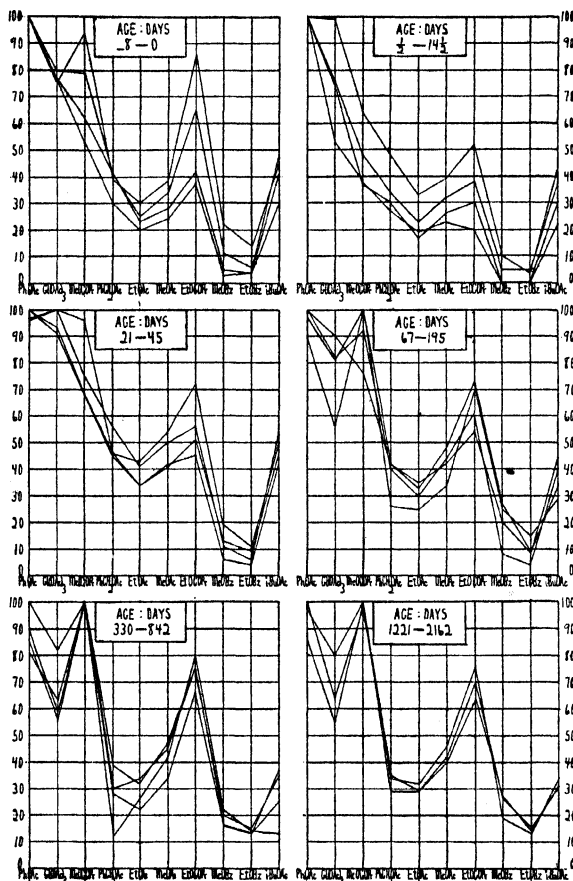
FIG. 5. *Leg Muscle Extracts.* As shown in the following table (Table V), eight of these series were obtained at a concentration of 17.8 mg. tissue per cc. of solution tested, one at 35.6 mg., fourteen at 44.4, and one (102 days) the average of the relative actions obtained at 17.8 mg. and 44.4 mg. The absolute actions were comparatively small at the lower concentration. This fact would increase the percentage error in the relative actions, and would account for the greater irregularities found with the curves in this figure. Even under these conditions, definite trends in the types or pictures may be observed. At the earliest ages studied, the types were considerably different from the embryo type although two of these curves (−3 days and 0 days) in the first group are somewhat similar to it. In the second group ($\frac{1}{2}$ to 14$\frac{1}{2}$ days) there is a real return to the embryo type. The drop in the actions on the butyrates and benzoates and the increase in glyceryl triacetate is unquestioned. Thereafter there is a development of the adult type which is apparently complete after about 3 months.

although some of the minor changes which take place appear to be reflected in small changes in the relative actions especially when pairs of esters only are considered, as pointed out in the discussion of the relative actions of these extracts.

TABLE V.

Hydrolyzing Actions in Tenths of Milli-Equivalents of Acid Produced by Leg Muscle Extracts of Rabbits of Different Ages on the Indicated Esters.

Age.	PhOAc	Gl(OAc)₃	MeOCOPr	PhCH₂OAc	EtOAc	MeOAc	EtOCOPr	MeOBz	EtOBz	i-BuOAc
days										
−8 (17.8)	0.71	0.57	0.56	0.29	0.18	0.24	0.46	0.08	0.04	0.34
−7 (17.8)	0.69	0.52	0.65	0.27	0.21	0.26	0.59	0.15	0.10	0.31
−3 (17.8)	0.79	0.60	0.42	0.24	0.16	0.19	0.29	0.02	0.03	0.24
0 (17.8)	0.78	0.68	0.48	0.32	0.18	0.22	0.33	0.04	0.03	0.32
½ (17.8)	0.54	0.40	0.20	0.16	0.09	0.14	0.16	0.00	0.00	0.12
2½ (17.8)	0.74	0.49	0.28	0.20	0.14	0.17	0.15	0.00	0.00	0.22
102 (17.8)	0.45	0.35	0.39	0.15	0.10	0.18	0.24	0.00	0.00	0.15
195 (17.8)	0.64	0.41	0.74	0.15	0.12	0.21	0.52	0.15	0.06	0.15
842 (17.8)	0.57	0.38	0.64	0.18	0.14	0.22	0.42	0.10	0.08	0.16
14½ (35.6)	1.01	1.00	0.65	0.48	0.33	0.39	0.53	0.10	0.04	0.43
6½ (44.4)	1.46	1.11	0.70	0.49	0.34	0.46	0.55	0.08	0.08	0.54
21 (44.4)	1.05	0.93	0.68	0.46	0.34	0.42	0.44	0.05	0.04	0.42
28 (44.4)	1.28	1.19	0.87	0.59	0.43	0.52	0.65	0.16	0.12	0.58
35 (44.4)	1.00	1.03	0.77	0.58	0.42	0.52	0.58	0.12	0.06	0.56
45 (44.4)	1.04	1.08	1.04	0.50	0.46	0.58	0.78	0.21	0.12	0.54
67 (44.4)	1.35	1.21	1.02	0.57	0.47	0.57	0.73	0.27	0.11	0.59
102 (44.4)	1.03	0.89	0.99	0.47	0.38	0.48	0.71	0.15	0.08	0.47
136 (44.4)	1.45	1.22	1.50	0.63	0.49	0.72	1.10	0.40	0.14	0.51
195 (44.4)	1.37	0.86	1.54	0.40	0.39	0.53	1.08	0.38	0.23	0.44
330 (44.4)	1.10	0.73	1.30	0.16	0.34	0.54	1.04	0.29	0.18	0.17
501 (44.4)	1.22	0.94	1.50	0.45	0.51	0.67	1.12	0.30	0.22	0.52
663 (44.4)	1.14	0.93	1.14	0.44	0.36	0.54	0.86	0.18	0.15	0.42
1221 (44.4)	1.15	0.95	1.19	0.41	0.38	0.55	0.91	0.21	0.16	0.41
1737 (44.4)	1.68	1.08	1.63	0.58	0.48	0.67	1.07	0.46	0.23	0.52
2162 (44.4)	1.33	0.84	1.54	0.44	0.44	0.64	1.08	0.40	0.23	0.47

In considering the physiological relations in connection with these enzyme actions, the striking fact is the great change which occurs at birth when breathing begins.

The absolute actions of the skin extracts are shown in Table IV.

The changes in the absolute actions with increase in the ages of the rabbits are small compared to those found with some of the other tis-
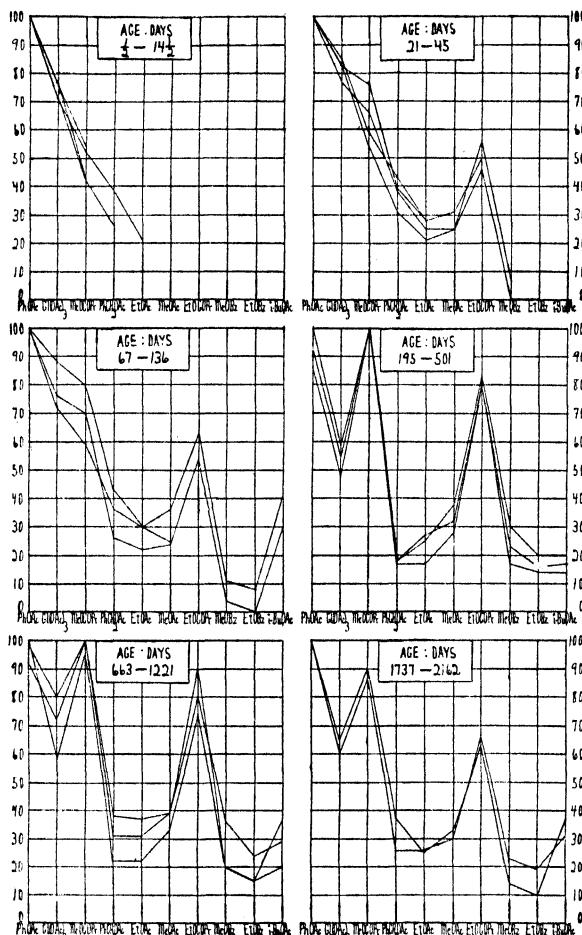
FIG. 6. *Heart Muscle Extracts.* The pictures for the youngest rabbits, as far as can be told from the few results obtained because of the small amount of material available, approach the embryonic type, diverging steadily as the rabbits become older until the adult type is reached at about 6 months. The relative increases in the butyrates and benzoates and decrease in glyceryl triacetate are noteworthy. There appears to be a real reversion for the oldest rabbits. Because of the comparatively small absolute actions, the percentage errors in the relative actions are magnified, and the resulting curves somewhat irregular.

sues. The results given refer to the concentration of 17.8 mg. skin per cc. of solution tested. The values for the acetates remained practically unchanged except that the glyceryl triacetates decreased after 4 months.   On the other hand, the values for the butyrates and benzoates increased markedly for the first few months and then remained constant.   In general, the actions on the methyl esters were greater than on the corresponding ethyl esters.

TABLE VI.

Hydrolyzing Actions in Tenths of Milli-Equivalents of Acid Produced by Heart Muscle Extracts of Rabbits of Different Ages on the Indicated Esters.

Age.	PhOAc	Gl(OAc)₃	MeOCOPr	PhCH₂OAc	EtOAc	MeOAc	EtOCOPr	MeOBz	EtOBz	i-BuOAc
days										
⅓ (dil.).	0.62	0.44	0.26	0.16						
2⅓	0.92	0.70	0.40							
6⅓	1.04	0.74	0.54	0.40	0.22					
14⅓	1.40	1.06	0.76							
21	0.96	0.80	0.52	0.30	0.20	0.24	0.44	0.00	0.00	
28	1.08	0.92	0.64	0.46	0.30	0.34	0.54			0.40
35	1.22	0.94	0.80	0.46	0.30	0.30	0.68	0.08		
45	0.92	0.76	0.70	0.36	0.26					
67	1.28	0.92	0.76	0.46	0.38	0.32				
102	0.92	0.70	0.64	0.24	0.20	0.22	0.50	0.04	0.00	0.28
136	1.15	0.01	0.92	0.49	0.35	0.41	0.73	0.13	0.09	0.47
195	0.58	0.34	0.58	0.10	0.10	0.16	0.46	0.10	0.08	0.08
330	0.69	0.38	0.80	0.14	0.20	0.30	0.66	0.24	0.16	0.16
501	0.82	0.48	0.88	0.16	0.24	0.28	0.70	0.20	0.14	0.15
663	0.80	0.66	0.82	0.31	0.30	0.32	0.74	0.16	0.12	0.30
842	0.81	0.47	0.77	0.18	0.18	0.27	0.60	0.16	0.12	0.16
1221	0.76	0.60	0.83	0.26	0.26	0.32	0.66	0.30	0.20	0.24
1737	1.00	0.65	0.90	0.37	0.25	0.33	0.63	0.14	0.10	0.37
2162	0.94	0.56	0.81	0.24	0.24	0.28	0.62	0.22	0.18	0.20

The absolute actions of the leg muscle extracts are shown in Table V.

The concentrations of the extracts in terms of mg. of tissue extracted per cc. of solution tested are given in parentheses in Column 1 following the ages of the rabbits.   The decrease in the actions on the butyrates and benzoates just before birth and during lactation, followed by the

increase after weaning were referred to in speaking of the relative actions. Beyond this the variations are irregular and point in no definite direction.

The absolute actions of the heart muscle extracts are shown in Table VI.

FIG. 7. *Brain Extracts*. The relative actions are shown, six on the first plot, five on each of the other three plots. There is practically no change in the type throughout the life cycle. There is a small apparent increase in the relative actions of the butyrates at the ages between 45 and 136 days but in view of the comparatively small absolute actions of the extracts, as shown in Table VII, this increase is not at all certain and may be due to the experimental inaccuracies.

The type of action, is in general, similar to the embryo type. Minor differences may be observed in the smaller actions of the brain extracts on the esters following phenyl acetate and glyceryl triacetate, in the actions on the benzoates which are practically zero, and in the actions on the isomers ethyl butyrate and isobutyl acetate which are essentially the same. The striking features of these actions may be found in the fact that they are similar in type to the actions of all brain extracts so far studied, including human, beef, and rat, and that they are similar to the embryo type, to the Flexner-Jobling rat carcinoma type, and to a number of tumors of human origin.

The action for the $\frac{1}{2}$ day old rabbit was obtained at the concentration of 8.9 mg. tissue per cc. of solution tested; the remaining actions were obtained at the 17.8 mg. concentration.

With phenyl acetate a small decrease in action was observable as the rabbits became older; with glyceryl triacetate the decrease was

TABLE VII.

Hydrolyzing Actions in Tenths of Milli-Equivalents of Acid Produced by Brain Extracts of Rabbits of Different Ages on the Indicated Esters.

Age.	PhOAc	Gl(OAc)₃	MeOCOPr	PhCH₂OAc	EtOAc	MeOAc	EtOCOPr	MeOBz	EtOBz	i-BuOAc
days										
−8 (dil.).	0.41	0.24	0.10	0.06	0.07	0.07	0.06	0.00	0.00	0.10
−7	0.78	0.48	0.29	0.17	0.12	0.18	0.22	0.00	0.00	0.19
$\frac{1}{2}$	0.73	0.43	0.18	0.15	0.12	0.16	0.12	0.00	0.00	0.16
$2\frac{1}{2}$	0.78	0.44	0.22	0.14	0.13	0.16	0.15	0.00	0.00	0.13
$6\frac{1}{2}$	0.90	0.43	0.24	0.18	0.13	0.19	0.17	0.00	0.00	0.17
$14\frac{1}{2}$	0.82	0.48	0.26	0.20	0.10	0.15	0.12	0.00	0.00	0.15
21	1.12	0.64	0.29	0.29	0.22	0.27	0.23	0.00	0.00	0.27
28	1.63	0.93	0.54	0.41	0.27	0.30	0.44	0.00	0.00	0.35
35	1.12	0.56	0.36	0.24	0.20	0.20	0.28	0.06	0.04	0.20
45	0.90	0.55	0.40	0.25	0.20	0.22	0.33	0.08	0.04	0.20
67	1.77	1.02	0.76	0.62	0.37	0.38	0.64	0.12	0.04	0.50
102	0.89	0.42	0.27	0.18	0.08	0.14	0.16	0.00	0.00	0.14
136	0.86	0.46	0.46	0.26	0.22	0.18	0.38	0.06	0.04	0.24
195	0.83	0.35	0.26	0.15	0.11	0.15	0.17	0.00	0.00	0.16
330	0.58	0.27	0.15	0.12	0.08	0.12	0.14	0.04	0.00	0.10
501	0.60	0.26	0.18	0.09	0.08	0.04	0.12	0.02	0.00	0.12
663	0.54	0.30	0.15	0.11	0.02	0.06	0.09	0.00	0.00	0.07
842	0.83	0.33	0.26	0.16	0.08	0.12	0.14	0.04	0.00	0.14
1221	1.51	0.64	0.31	0.26	0.20	0.20	0.28	0.00	0.00	0.25
1737	1.47	0.71	0.51	0.34	0.21	0.27	0.32	0.09	0.05	0.28
2162	0.75	0.40	0.29	0.14	0.10	0.14	0.18	0.04	0.00	0.16

very marked.   The action on methyl butyrate increased with increase in age; that on ethyl butyrate increased to a small extent.   On benzyl acetate, there was a small but distinct decrease.   No definite changes could be determined with the remaining esters, possibly because of the small actions.

Heart activity increases as the young animal becomes more active;

at the age of 2 weeks approximately, the animals are able to move about. Before then the motions are more or less uncoordinated. The pictures of the relative actions reflect these changes to a certain extent. It is possible that the decreases in some of the absolute enzyme actions beginning in the early adult life may have some physiological significance.

The absolute actions of the brain extracts are shown in Table VII.

FIG. 8. *Spleen Extracts.* Five curves are shown on the first plot and four on each of the succeeding. The pictures resemble in general those of the embryo, except that, as with the brain extracts the relative values on the benzoates are very small. There is no real change in type at the different ages.

The first results (−8 days) refer to a concentration of 8.9 mg. tissue per cc. of solution tested, the remaining results to 17.8 mg. per cc.

The interesting feature of these absolute actions is that there is no regular change observable. The actions are practically of the same order of magnitude for each ester throughout. Several series show somewhat greater actions on all the esters, notably those of 28, 67, 1221, and 1737 days of age, but these are isolated and cannot be taken

as indicative of a regular trend. As stated before, such irregularities are to be expected when dealing with biological material.

The absolute values of the spleen extracts are shown in Table VIII.

Because of their small size, it was not possible to study the actions of the spleens in the embryos. The concentrations of the extracts which were tested corresponded to 2.2 mg. tissue per cc. of solution

TABLE VIII.

Hydrolyzing Actions in Tenths of Milli-Equivalents of Acid Produced by Spleen Extracts of Rabbits of Different Ages on the Indicated. Esters.

Age.	PhOAc	Gl(OAc)₃	MeOCOPr	PhCH₂OAc	EtOAc	MeOAc	EtOCOPr	MeOBz	EtOBz	i-BuOAc
days										
2½	0.42	0.32	0.20	0.06	0.04	0.04	0.16	0.00	0.00	0.08
6½	0.60	0.32	0.32	0.20	0.12	0.12	0.28	0.00	0.00	0.16
14½	1.40	0.92	0.65	0.56	0.30	0.28	0.65	0.00	0.00	0.51
21	1.30	0.89	0.65	0.41	0.27	0.25	0.57	0.02	0.00	0.37
28	3.06	1.66	1.00	0.76	0.54	0.48	0.96	0.08	0.04	0.70
35	1.78	0.99	0.64	0.42	0.25	0.24	0.53	0.06	0.00	0.36
45	1.61	1.04	0.69	0.46	0.24	0.30	0.66	0.04	0.00	0.44
67	2.14	1.01	0.60	0.38	0.31	0.29	0.62	0.04	0.01	0.41
102	3.66	1.97	1.18	0.64	0.42	0.38	0.08	0.06	0.02	0.64
136	1.75	0.86	0.54	0.38	0.30	0.26	0.52	0.04	0.02	0.38
195	3.38	1.39	0.83	0.36	0.20	0.26	0.70	0.08	0.04	0.35
330	1.15	0.71	0.69	0.27	0.21	0.19	0.56	0.08	0.08	0.36
501	1.98	0.98	0.68	0.22	0.11	0.16	0.54	0.03	0.02	0.14
663	1.07	0.71	0.54	0.33	0.17	0.16	0.48	0.02	0.00	0.29
842	1.17	0.50	0.35	0.14	0.10	0.08	0.30	0.04	0.00	0.12
1221	2.44	1.21	0.64	0.34	0.22	0.26	0.53	0.00	0.00	0.33
1737	1.20	0.69	0.60	0.34	0.24	0.23	0.51	0.06	0.04	0.34
2162	2.10	0.77	0.64	0.23	0.16	0.16	0.50	0.06	0.04	0.16

tested. This dilution was much greater than with any other tissue studied. In view of this fact, the enzyme actions found may be said to be quite large. This is of special significance in comparing the brain and spleen actions. The types with these two tissues are very much the same (Figs. 7 and 8), but the absolute actions of the spleens are so much greater that no possibility of confusion exists.

As for the absolute actions of the spleen extracts at the different ages, the actions for the 2½ days and 6½ days rabbits are comparatively
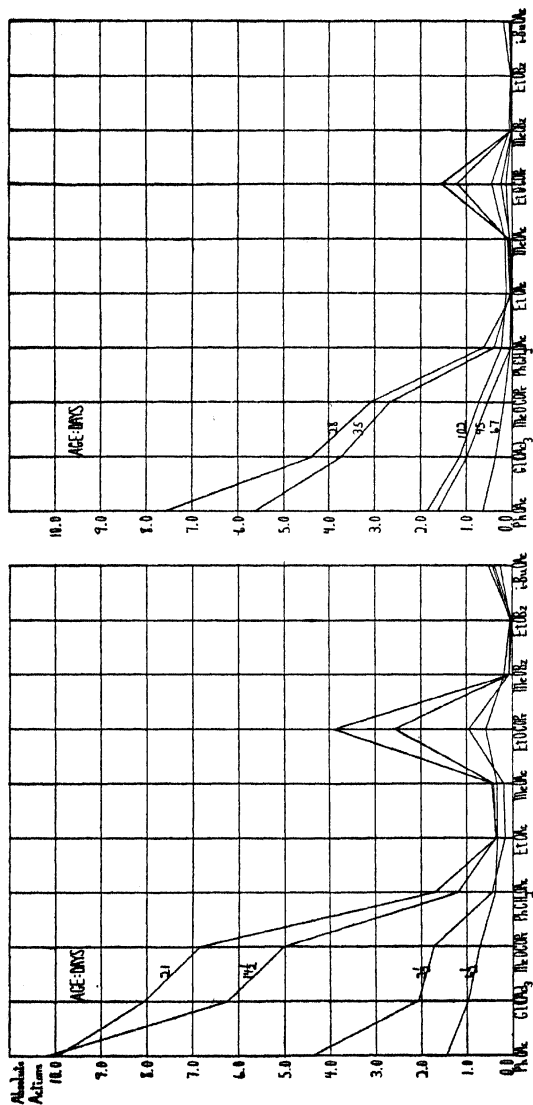
Fig. 9. *Stomach Extracts.* These enzyme actions present such an entirely different character that they will be presented in a manner different from the other tissues considered. The absolute actions of these extracts referred to the content of 17.8 mg. tissue per cc. solution are shown. The esters are arranged in the usual order at equidistant intervals along the abscissa axis; the actions corrected for blanks are given as ordinates. The ages of the rabbits are marked on the curves. Four curves are given in the first plot, five in the second.

The striking feature of these results is the increase in actions with the first four esters and ethyl butyrate reaching a maximum at 14 to 28 days and then decreasing again. After the age of 102 days, the actions of the stomach extracts are practically zero for all the esters. While the actions do not increase and decrease in mathematical order as compared to the ages of the rabbits, the general trend is clear and the large actions on certain of the esters, followed by the disappearance of the actions is very definite. The general type of the actions can be seen from the curves although a comparison of the type at the different ages is difficult because of the different magnitudes of the actions. In general, the lack of action on methyl, ethyl and isobutyl acetates, and on the benzoates is significant. In several series of experiments, the enzyme tests were carried out starting at pH 5.0 instead of pH 7.0. The results obtained were similar, so that the actions found do not appear to be due to the hydrogen ion concentration.

small, increasing quite rapidly thereafter.   For the first five esters the actions appear to reach a maximum at about the time of sexual maturity, decreasing somewhat thereafter.   This trend is clear in spite of certain individual irregularities, especially with the acetates.   The actions on the benzoates are practically zero throughout.

In connection with these results it may be of interest to point out that the rabbit spleen is readily autotransplantable in the young rabbit but not in the adult.[5]   This is an indication that the spleen is more important to the young rabbit than to the old.

The stomach results raise some interesting physiological questions. In the first place, the study of the ester-hydrolyzing enzymes of the stomach has given at times contradictory results.   If the conclusions obtained with rabbits apply to other animals, it is possible that some of the contradictions may be explained as due to the different ages of the animals studied.   This age factor has been found to be important with most of the tissues of the rabbit as shown in this paper, but in no case has its significance been as striking as with the stomach.   At the same time it must be remembered that there may be a difference in testing the stomach contents and testing the extract of the stomach. The actions found with the nursing rabbits as compared with the adult are of interest in relation to the food consumed, mainly milk with the former, very little or no milk with the latter.   If similar enzyme relations were found to hold in man, it would prove of considerable importance in infant nutrition.

Small Intestine Extracts.—The intestinal tract was divided into three parts (small intestine, appendix, and large intestine) and their ester-hydrolyzing actions determined in the usual way.   The absolute results for the extracts of the appendix and large intestines were very irregular and the pictures of the relative actions while not as irregular as the absolute actions do not show any definite types or changes. They will therefore not be included here and only the results for the small intestine given.   The types for the relative actions of the small intestine are in a measure similar to the embryo type but show greater general actions.   The results for the butyrates are markedly greater than with the embryo type.   The type does not change during the

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[5] Marine, D., and Manley, O. T., *J. Exp. Med.*, 1920, xxxii, 113.

life cycle of the rabbit as far as these results go. For this reason, the curves showing the relative actions will be omitted and only the absolute action shown (Table IX).

These actions refer to the concentration of 17.8 mg. per cc. of solution tested. There appears to be a definite increase in the values throughout beginning with the 14th day, coincident with the change

TABLE IX.

Hydrolyzing Actions in Tenths of Milli-Equivalents of Acid Produced by Extracts of the Small Intestine of Rabbits of Different Ages on the Indicated Esters.

Age.	PhOAc	Gl(OAc)₂	MeOCOPr	PhCH₂OAc	EtOAc	MeOAc	EtOCOPr	MeOBz	EtOBz	i-BuOAc
days										
2½	2.06	0.82	0.88	0.38	0.28	0.32	0.68	0.14		0.42
6½	1.90	0.96	0.69	0.50	0.32	0.28	0.59	0.14	0.02	0.40
14½	3.09	1.67	1.14	0.76	0.60	0.55	0.95	0.27	0.17	0.77
21	2.71	1.84	1.49	1.01	0.78	0.70	1.38	0.32	0.23	1.00
28	3.09	1.93	1.38	1.15	0.89	0.76	1.36	0.32	0.28	1.09
35	3.62	2.31	1.77	1.23	0.94	0.86	1.61	0.41	0.26	1.13
45	3.43	2.28	1.50	1.18	0.92	0.82	1.45	0.38	0.28	1.12
67	2.59	1.57	1.11	0.93	0.70	0.60	1.09	0.29	0.16	0.86
102	3.69	2.25	1.81	1.02	0.86	0.74	1.53	0.34	0.26	1.02
136	2.90	1.84	1.36	1.13	0.82	0.77	1.27	0.34	0.24	1.03
195	3.10	1.88	1.68	0.85	0.63	0.57	1.45	0.29	0.23	0.82
330	2.51	1.27	0.85	0.37	0.32	0.31	0.80	0.16	0.12	0.46
501	3.22	1.88	1.57	0.80	0.63	0.60	1.32	0.33	0.24	0.77
663	2.48	1.69	1.27	0.80	0.62	0.53	1.15	0.21	0.14	0.79
842	2.75	1.52	1.37	0.73	0.55	0.53	1.24	0.30	0.27	0.69
1221	3.28	1.95	1.34	0.85	0.71	0.65	1.14	0.32	0.22	0.85
1737	1.74	0.90	0.68	0.44	0.30	0.26	0.60	0.14	0.12	0.38
2162	2.66	1.41	1.11	0.58	0.41	0.40	1.04	0.20	0.18	0.57

from milk to a mixed dietary. The values then remain fairly constant except for the usual irregularities until with the oldest rabbits the actions appear to decrease somewhat.

In order to show more clearly the relation between different tissues at the same ages, the results brought together in Fig. 10 are presented.

The comparative results of the different tissues, as shown in Fig. 10, may be summarized briefly. The brain does not change in type but

remains embryonic throughout.   The liver, between the ages studied, does not show the embryonic type at all.   It seems to show the adult type throughout the life cycle except for the reversion in the direction of the embryonic type during the first 4 weeks of life.   This reversion,
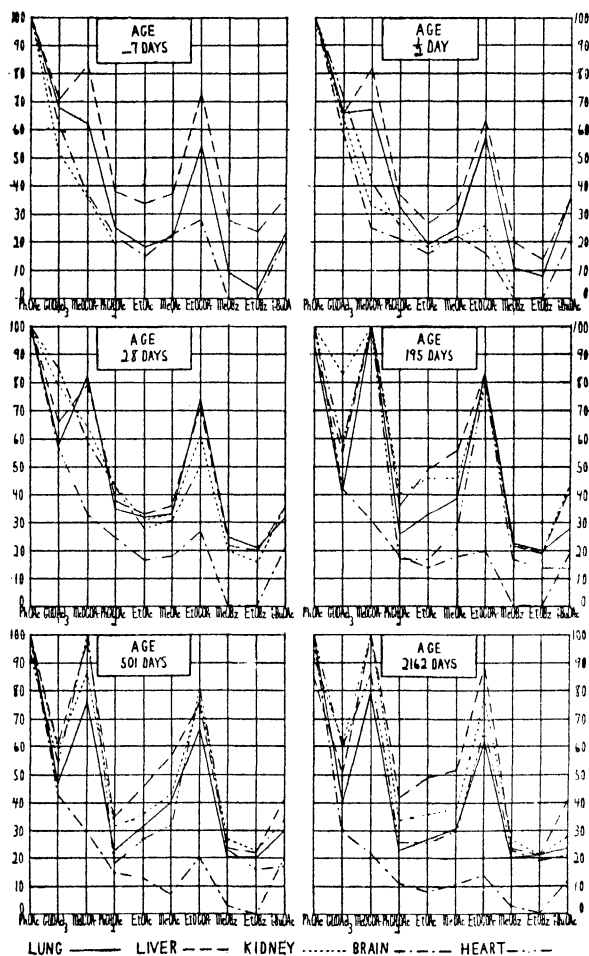
FIG. 10. Relative lipase actions of extracts of lung, liver, kidney, brain, and heart muscle of rabbits of different ages.   Six different ages are shown, one age to a plot.   The ages chosen are −7 days (embryonic), ¼ day (after birth), 28 days (dietary fairly mixed, but rabbits not weaned), 195 days and 501 days (adult and sexually mature), 2162 days (old age).

however, is not enough to overshadow the adult type of action. The kidney clearly shows the embryonic type in the first two plots, gradually changing (28 days), the change being completed in the adult stages. The lung on the other hand, while being close to the embryo type in the first plot (a series obtained at -8 days shown in Fig. 3 but not given in Fig. 10 indicates the embryo type very clearly) changing rapidly and becoming adult in type at or before 28 days. The heart muscle was embryonic in type at $\frac{1}{2}$ day, the change to adult being only partial at 28 days, but complete at 195 days.

Perhaps the most striking feature of these results is to be found in the fact that different tissues lose their embryonic character as shown by the enzyme actions at different periods of life. In general, those tissues that function least in embryonic life show by their enzyme actions the greatest similarity to the embryo type as well as to certain tumor types. These would include the kidney, skin, and lung. On the other hand the liver, which functions actively in the rabbit embryo behaves chemically as the adult type. Without entering into greater detail, it may be said that there is a striking correlation between the chemical evidence based upon the ester-hydrolyzing enzymes and the functional activity with rabbit tissues.

The question of reversion from the adult type as the rabbit becomes older is of interest. With the whole rat the reversion of the type from the adult picture toward a picture approaching the younger rats, even if not attaining the embryo character was clear and unmistakable. With the rabbit tissues such reversions were not as marked. This may have been due to the ages of the animals. It was possible to obtain rats of extreme old age (i.e., in the neighborhood of three years of age). On the other hand, the oldest rabbits which were available, were five to six years old. If still older rabbits could be secured, say up to eight years of age, the results might be more complete and satisfactory. Even with the rabbits aged five to six years, definite reversions in types and in absolute actions were clearly apparent, although the changes were not as great as with the whole rats. These reversions with the rabbit tissues were given in the consideration of the separate tissues. The results are hardly complete enough to warrant careful comparison and a study of the ages at which the different tissues show such reversion. Except for this brief reference, therefore, these relations will not be considered farther in this connection.

SUMMARY.

The ester-hydrolyzing actions of extracts of a number of tissues of rabbits of different ages were studied under comparable conditions.

The ages of the rabbits ranged from 8 days before birth to 2162 days. The esters used included phenyl acetate, glyceryl triacetate, methyl butyrate, benzyl acetate, methyl acetate, ethyl acetate, ethyl butyrate, methyl benzoate, ethyl benzoate, and isobutyl acetate. The following tissues were studied: kidney, liver, lung, skin, leg muscle, heart muscle, brain, spleen, stomach, and small intestine.

The results, as in previous communications, are presented in the form of plots for the relative enzyme actions, and in tables for the absolute actions.

The changes in the curves of the relative actions as the rabbits became older are considered in some detail. The relations between the embryonic state of certain tissues, as shown by their enzyme actions, and the adult state, are described, and compared with their physiological behavior. The probable reversion to a type approaching the embryonic for the oldest rabbits studied is indicated with some of the tissues. The changes in the absolute enzyme actions of the tissues as the rabbits became older are also discussed. The absolute actions do not form as regular a progression as do the relative actions but, at the same time, show marked regularities with increasing age of the rabbits.

MOLECULAR STRUCTURE OF PLANT FIBERS DETERMINED BY X-RAYS.

By O. L. SPONSLER.

(From the University of California, Southern Branch, Los Angeles.)

In an earlier paper[1] a method of photographing x-ray diffraction patterns from plant fibers, ramie fibers especially, was discussed, and the data were reported for some thirty lines. It was shown there that certain sets of planes of atoms, the existence of which was indicated by the diffraction lines, extended lengthwise of the fibers, while other sets were transverse and still others occurred diagonally in the fiber. The presence of these planes postulates the existence of a three dimensional lattice whose elementary cell was interpreted to be an orthorhombic structure.

The longitudinal planes were considered when discussing the diffraction patterns from the 0° position of the fiber block.[1] A study of the transverse and the diagonal planes is undertaken in the present paper. In addition to that, the character of the unit groups and their orientation with respect to one another will be considered here.

Transverse Planes.

The data given in the earlier paper for the diffraction pattern from the 90° position of the block (Fig. 1) showed that eight lines seemed to have been produced by as many sets of planes, *all* of which were parallel to one another. In attempting to interpret these lines two explanations seemed possible; either the lines were produced by reflection of higher orders from a single set of widely spaced planes, or they were produced by several sets of planes all parallel to one another. In either case there must exist a simple multiple relation between the values of the interplanar spacings such as is shown in Table I, Columns 1 to 3.

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[1] Sponsler, O. L., *J. Gen. Physiol.*, 1925–26, ix, 221.

The first alternative does not seem to satisfy the conditions.   In work with well defined crystals, lines such as these would be suggestive of higher orders where n, in the fundamental formula $n \lambda =$
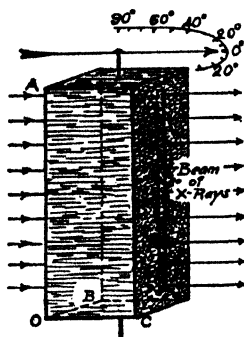
FIG. 1. Block of fibers set at 0° position. Fibers parallel to *OC*. Path of beam of x-ray is indicated by arrows.   This figure appeared in the preceding paper.[1]

$2d \sin \theta$, is equal to 2 or 3 or some higher whole number.   Here, however, we are dealing, not with well defined crystals, but with a mass of perhaps 10,000 small fibers which do not possess ordinary

TABLE I.

Transverse Planes.

n	Observed interplanar spacing d.	$n \times d$	Calculated spacing based on average $n \times d = 10.25$.	Crystallographic indices from Fig 3.
	Å.u.		*Å.u.*	
1	—	—	10.25	001
2	5.15 s.	10.30	5.13	002
3	3.40 m.	10.20	3.42	003
4	2.58 vvs.	10.32	2.56	004
5	2.03 s.	10.15	2.05	005
6	1.70 s.	10.20	1.71	006
7	1.46 vw.	10.22	1.46	007
8	1.29 w.	10.32	1.28	008
9	1.14 w.	10.26	1.14	009

vvs., exceptionally strong; s., strong; m., medium; w., weak; vw., very weak.

crystal characteristics.   Nowhere in our work with fibers have we found evidence of higher order reflections from a single set of planes,

beyond the second order, and then the lines were very weak. The possibility of obtaining sixth, seventh, and eighth orders, or even third and fourth, from a single set of widely spaced planes from material of this kind, and with the exposures such as used in this work, is indeed very remote. Further, the density of the lines does not, by any means, agree with the relative intensity of reflection from higher orders. This lack of agreement is brought out in Table II where it may be noted that the fourth order from rock-salt is about *one-twentieth* as strong as the second order, while the apparent fourth order from fibers is fully *three times* as strong as the second order.

The other alternative, that each diffraction line represents a specific set of planes, seems much more probable. On that assumption

TABLE II.

Intensity of Reflection of Higher Orders.

	Percentage based on strongest line.		
Order of reflection.	(100) face NaCl.* Density.	Fibers. Estimated density.	Interplanar spacing of fibers from Table I.
	per cent	*per cent*	*Å.u.*
1	100	—	—
2	20	30	5.15
3	5	10	3.40
4	1	100	2.58
5	0.1	30	2.03

*Approximate figures from Bragg, W. H., and Bragg, W. L., X-rays and crystal structure, London, 4th edition, 1924, 198–202.

there would be several, perhaps eight, sets of parallel transverse planes in the fiber. One set would have its planes spaced about 10.25 Å.u. apart; a second set would consist of the same planes with a set interleaved half way between; that is, 5.13 Å.u. apart; a third set would be composed of those spaced 10.25 along with a set interleaved by thirds; that is 3.42 Å.u. apart, and so on. This may be more clearly seen in Fig. 2 where the lines A_1 and A_2 represent identical transverse planes in a fiber with the spacing of 10.25 Å.u. The lines between indicate the positions of the interior planes which interleave them, and the figures denote the interplanar spacing. The

structure would represent an arrangement of transverse planes which would be repeated from one end of the fiber to the other.

The principal evidence supporting this assumption lies in the relative intensities of the lines. By referring to Table I, Column 2, it will be noticed that the 10.25 line is missing, that the 5.15 line is moderately strong and that the 2.58 line is exceptionally strong. The great density of the latter, for it is the strongest line produced by the fibers on any diffraction pattern except that from the 0° position, practically forces our recognition of a set of 2.58 planes. If these 2.58 planes are alternately light and heavy planes, that is containing few and many atoms per unit of area respectively, then a weaker 5.15 line would appear. Its density would depend upon the difference in the number of atoms in the planes. The like planes would then be spaced 5.15 Å.u., but if they in turn were alternately light

A_1 ——————— 10.25 ‑‑ 5.13 ‑‑ 2.56 ‑‑ 3.42 ‑ 1.71

B' ══════════ .... ‑‑‑‑‑‑‑‑‑ 1.71
‑‑‑‑‑‑‑‑‑‑‑‑‑‑‑‑‑‑‑‑‑‑‑‑ 2.56
.... 3.42 ‑ 1.71

A'_1 ——————— ‑‑‑‑‑‑‑‑‑ 5.13 ‑‑ 2.56 ‑‑‑‑‑‑‑‑ 1.71

B'' ══════════ .... 3.42 ‑ 1.71
‑‑‑‑‑‑‑‑‑‑‑‑‑‑‑‑‑‑‑‑‑‑‑ 2.56
.... 1.71

A_2 ——————— ‑ 10.25 ‑‑ 5.13 ‑‑ 2.56 ‑ 3.42 ‑ 1.71
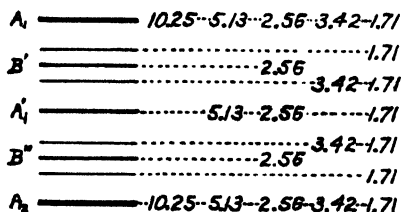
FIG. 2.

and heavy, the like planes then would be 10.30 Å.u., and a weaker line representing those planes might be expected to appear on the diffraction pattern. Lines from such wide spacings, however, are quite likely to be obscured in the general fog from the principal beam where moderately short wave-lengths of x-rays are being used, unless extremely well formed crystal materials are being photographed. The lines lettered A_1, B', A'_1, B'', and A_2 represent 2.56 planes. The diffraction line from these planes would be produced by an additive effect resulting in a strong line; but when the A planes are in position to produce a 5.13 line the B planes would partially annul the reflection and the resulting line would be weaker. Again when the widely spaced planes A_1 and A_2 are in position to produce a 10.25 line, the A'_1 plane would be effective in partially annulling the reflection, and a still weaker line would be the result When the 1.71

planes are considered the explanation becomes still more complicated but in general is of a similar nature.

The production of the lines on the photographic film is very probably still more involved because second order reflections may tend to make them denser in some cases; for example the 2.58 line may be reinforced by the second order of 5.15. On the other hand, the 1.29 line may be second order only, of the 2.58.

One might infer that atoms occur between the 1.71 planes, because the appearance of a 2.03 line indicates the existence of planes located at one-fifth intervals between the A_1 and A_2 planes.

On the whole the evidence strongly favors the conclusion that several sets of parallel transverse planes exist, in which the like planes are spaced about 10.25 Å.u. apart, and the other planes are interleaved at certain intervals between them.

That arrangement of planes indicates a structural unit whose length is equal to the distance between the like planes; and a unit which is repeated lengthwise of the fiber.

The Structural Unit.

In the earlier paper[1] it was shown that the dimensions of this unit on a cross-section of the fiber are 6.10 Å.u. $\times$ 5.40 Å.u. Now a point is reached where we may say that the other dimension of the elementary cell is about 10.30 Å.u. and its volume is about 340 cu. Å.u.

$$6.10 \times 5.40 \times 10.30 = 339.$$

One might surmise that this volume would bear a direct relation to the volume of a $C_6H_{10}O_5$ group since the chemist has shown that cellulose is very probably made up structurally of anhydroglucose residues.[2] The volume of one $C_6H_{10}O_5$ group based on the specific gravity of ramie fibers,[3] which are almost pure cellulose, is 170 cu. Å.u.

$$\frac{162.1}{1.57} \times \frac{1}{6.062 \times 10^{23}} = 170 \times 10^{-24} \text{ cm.}^3 \text{ or } 170 \text{ cu. Å.u.}$$

[2] Irvine, J. C., *Chem. Rev.*, 1924, i, 55.

[3] de Mosenthal, H., *J. Soc. Chem. Ind.*, 1907, xxvi, 443.

The sum of the atomic weights for the $C_6H_{10}O_5$ group is 162.1. The figure 6.062×10^{23} is Avogadro's number. The volume of the elementary cell then is equal to that of two of the glucose residues.

The two C_6 groups placed in Fig. 2 would have certain atoms in plane A_1. The atoms in A_2 would be the corresponding atoms of a second pair of C_6 groups; those in A_3, if that were shown, would be the same for a third pair of C_6 groups and so on, forming a chain of such groups. On each side of this chain would occur other parallel chains 6.10 Å.u. apart in one direction and 5.40 Å.u. at right angles to that direction.

The arrangement of the transverse planes as interpreted in Fig. 2 would indicate that the C_6 groups are spread out lengthwise of the fiber to form unbroken chains of atoms; that is, there would be no distinct gaps between the groups. On the other hand, the longitudinal planes, as interpreted from the diffraction pattern from the 0° position of the fiber block[1] would indicate that the atoms are located close to the center line of the chain of groups with few if any of the heavier atoms occupying positions half way between the chains. Such a structure will account for the lines obtained from the 0° and the 90° positions (Fig. 1). If that structure is the correct interpretation it must also account for the lines obtained from positions intermediate between 0° and 90°.

Diagonal Planes.

In the earlier paper[1] there was given a list of interplanar values which were determined from photographs taken at intervals between the 0° and the 90° positions of the fiber block (see Fig. 1). The planes represented by them, which we will call diagonal planes, belong to the (111) series *OEG*; the (101) series, *OEHB*, and to the (011) series, *OAHG*, of Fig. 3. The figure represents an orthorhombic elementary cell where

$$OA:OB:OC = 5.40:6.10:10.30$$

and where *OC* is parallel to the long axis of the fiber. The planes *OADB* and *CEHG* are those represented in Fig. 2 by the lines A_1 and A_2. The planes lying between them are omitted for convenience.

A study of these diagonal planes resolves itself into: first, a sorting

out of the observed interplanar values to fit the calculated values of the possible planes of these series; and then, an assembling of the evidence to gain some further conception of the unit group of atoms.

When the fiber block (Fig. 1) was turned to some position, as at 60°,
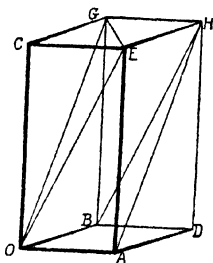
FIG. 3.

several representative sets of planes from the three series (111), (101), and (011) could readily have been oriented so as to produce their respective lines on the photographic film, all at the same time. This situation would be due to the combined effect of the cylindrical shape

TABLE III.

Planes from 60° Position of Fiber Block.

Interplanar spacing.		Angle to *OC* edge of fiber block, Fig. 1.		Crystallographic indices.	Density of lines.
Calculated.	Observed.	Calculated.	Observed.		
2.60	2.62	57°	60°	113	s.
4.31	4.35	61°	60°	114	vs.
2.16	2.17	66°	60°	114	s.
1.83	1.82	73°	60°	115	vw.
2.32	2.35	73°	60° and 70°	104	w.
2.94	2.97	68°	60° and 70°	013	s.

vs., very strong; s., strong; vw., very weak; w., weak.

of the fibers and the lack of exact parallelism in the orientation of the fibers in the block. As a result it would not be possible to determine *directly* with which of the three series of planes a given line on the negative would be associated. If, however, all of the lines were

TABLE IV.

(101) Series of Planes, OEHB, etc. of Fig. 3.

Crystallographic indices.	Interplanar spacing.		Angle to OC edge of fiber block, Fig. 1.		Density of lines.
	Calculated.	Observed.	Calculated.	Observed.	
	$\AA.u.$	$\AA.u.$			
100	5.35	5.40	4°	0°	s.
101	4.78	2.34	32°	20°	vvw.
102	3.92	3.95	48°	40°–50°	vw.
103	2.88	—	65°	—	—
104	2.32	2.35	73°	70°	w.
105	1.92	1.94	79°	70°–80°	vvw.
106	1.63	—	85°	—	—
107	1.41	1.44	89°	80°–90°	vvw.
108	1.25	1.25	93°	90°–100°	vw.
109	1.11	1.10	96°	90°–100°	w.
201	2.61	2.65	22°	20°	w.
301	1.78	—	21°	—	—

s., strong; w., weak; vw., very weak; vvw., extremely weak.

TABLE V.

(011) Series of Planes, OAHG, etc. of Fig. 3.

Crystallographic indices.	Interplanar spacing.		Angle to OC edge of fiber block, Fig. 1.		Density of lines.
	Calculated.	Observed.	Calculated.	Observed.	
	$\AA.u.$	$\AA.u.$			
010	6.05	6.10	3°	0°	vs.
011	5.25	2.65	38°	30°–40°	w.
012	3.93	3.95	55°	50°	vw.
013	2.94	2.97	68°	60°–70°	s.
014	2.36	2.35	75°	70°	w.
015	1.94	1.94	81°	80°	vvw.
016	1.64	1.69	87°	80°	w.
017	1.43	1.44	90°	80°–90°	vw.
018	1.25	1.25	94°	90°–100°	w.
019	1.12	1.10	98°	90°–100°	w.
021	2.92	2.93	20°	30°	vw.
031	1.78	—	21°	—	—

vs., very strong; s., strong; w., weak; vw., very weak; vvw., extremely weak.

in agreement with those which were predicted to appear at a given position, it would seem to be a safe procedure to ascribe certain

TABLE VI.

(111) Series of Planes, OEG, etc. of Fig. 3.

Crystallographic indices.	Interplanar spacing.		Angle to OC edge of fiber block, Fig. 1.		Density of lines.
	Calculated.	Observed.	Calculated.	Observed.	
111	3.72	1.88	31°	30°–40°	w.
112	6.28	6.40	40°	40°	vw.
	3.14	3.10	44°	30°–40°	vw.
	1.57	1.55	50°	40°	vw.
113	2.60	2.62	57°	60°	vs.
114	4.31	4.35	61°	60°	vs.
	2.16	2.17	66°	60°	s.
115	1.83	1.82	73°	60°	vw.
116	3.14	3.20	73°	70°	s.
	1.57	—	80°	—	—
117	1.38	—	84°	—	—
118	2.44	—	80°	—	—
	1.22	1.25	89°	80°–90°	vw.
119	1.10	1.10	93°	90°–100°	vw.
122	2.36	2.35	73°	60°–70°	w.
133	1.66	1.69	84°	80°–90°	w.
144	1.27	1.26	92°	90°	vw.
155	1.03	—	99°	—	—
221	1.96	1.98	21°	0°–10°	vw.
331	1.32	—	20°	—	—

vs., very strong; s., strong; w., weak; vw., very weak.

planes to particular lines and thus sort them out into their respective series. For example, the photograph taken at the 60° setting had on it the lines given in Column 2 of Table III. In the list of possible

planes computed from the elementary cell, there occurred planes with the spacings recorded in the first column of Table III. They formed angles to the *OC* edge of the fiber block as given in the third column. In most cases no attempt was made to estimate the observed angle closer than the interval reading[1] which was 10°. When considering the accuracy of agreement and when attempting to visualize the diffraction occurring in the mass of fibers during the formation of the six lines of Table III, one must keep in mind that a fiber, or at least that particular part of a fiber which is effective in reflecting to a given line, might not be oriented properly to be effective

TABLE VII.

(110) Series of Planes, ODHC, etc. of Fig. 3.

Crystallographic indices.	Interplanar spacing.		Angle to *OC* edge of fiber block, Fig. 1.		Density of lines.
	Calculated.	Observed.	Calculated.	Observed.	
100	5.35	5.40	4°	0°	m.
010	6.05	6.10	3°	0°	s.
110	{4.00	3.98	5°	0°	vs.
	2.00	1.98	10°	0°	vw.
120	2.65	2.65	7°	0°–10°	w.
130	1.90	1.93	10°	0°	vw.
210	2.46	—	7°	—	—
310	1.73	—	10°	—	—

vs., very strong; s., strong; m., medium; w., weak; vw., very weak.

in producing any other line. In other words each line would be associated with its own set of fibers or parts of fibers.

The results of sorting out the observed and the calculated planes are given in Tables IV, V, and VI. In order to complete the crystallographic data Table VII is also included.

Inspection of the tables shows fair agreement between the calculated and the observed values for the interplanar spacings and also for the position of the planes in the fiber, as based upon the elementary cell represented by Fig. 3.

Further agreement is found when a study of the densities of the lines is made. This evidence, however, should not be accepted at

its full face value because in a block of fibers the arrangement and distribution of the planes make it quite probable that certain lines appearing on the photographic film are composite lines. There is little doubt that some of the lines were produced by an additive effect of superimposed reflections from more than one set of planes. For example, the 2.35 line on the 60° and the 70° films might have been readily produced by reflections from the (104), the (014), and the (122) planes superimposed upon one another. While that situation must be taken into consideration it does not seem probable that very many lines fall in that category, and if comparisons between the densities are not used too specifically they would be acceptable as evidence.

TABLE VIII.

Comparison of Densities. From Tables IV, V, and VI.

Planes.	Density of lines.	Planes.	Density of lines.
101	vvw.	104	w.
102	vw.		
011	w.	013	s.
012	vw.	014	w.
111	w.	113	vs.
112	vw.	114	s.
		116	s.

vvw., extremely weak; vw., very weak; w., weak; s., strong; vs., very strong.

A comparison of the densities of certain lines from each of the three series seems to indicate again the presence of interleaved transverse planes, corroborating the conclusions previously made. A glance at these three tables (IV, V, and VI), shows that the densities of the lines have not decreased in the so called higher orders as one might have expected if the atoms of the groups had been closely segregated around the *corners* of the elementary cell (Fig. 3). The great density of the planes (113), (114), etc. as brought together in Table VIII, Columns 3 and 4, indicates the presence of atoms at various distances between the corners C and O, E and A, etc. The low intensity of reflection from the planes (101), (102), (011), etc.,

in Columns 1 and 2, indicates the presence of atoms half way between the 10.25 planes (*OADB* and *CEHG*).

From a somewhat different viewpoint, more information concerning the unit group may be obtained. In Tables IV, V, and VI there occur in several places, observed values of the interplanar spacings which are either one-half or double the calculated values. Such figures are indicative of interleaved planes and of alternating light and heavy planes. From work done with rock-salt and potassium chloride crystals[4] it has been shown that when the planes

TABLE IX.

Planes.	Interplanar spacing.		Density of lines.
	Calculated.	Observed.	
101	4.78	2.34	vvw.
011	5.25	2.65	w.
111	3.72	1.88	w.
112	6.28	6.40	vw.
	3.14	3.10	vw.
	1.57	1.55	vw.
114	4.31	4.35	vs.
	2.16	2.17	s.
116	3.14	3.20	s.
	1.57	—	—

vvw., extremely weak; vw., very weak; w., weak; s., strong; vs., very strong.

of a given set are unequal in reflecting power; that is, if they are alternately light and heavy planes, the first order lines are weaker than the second order. In other words, the reflection from the heavy planes is partly annulled by that from the light planes. The more nearly the planes approach equality in reflecting power, the fainter are the first order lines. The limit is reached when the planes are all equal; then, the first order line fails to appear. In those crystals a "heavy" plane consists of heavy atoms and a "light" plane of

[4] Bragg, W. H., and Bragg, W. L., X-rays and crystal structure, London, 4th edition, 1924, 92.

atoms with lower atomic weights. In the fibers, however, the carbon and oxygen atoms are so nearly alike in weight that a "heavy" plane is quite likely to be one in which there are more atoms per unit area than occur in a "light" plane.

In Table IX examples of these two types of spacings are brought together from the preceding tables. The first three values indicate the presence of atoms on the vertical lines of Fig. 3, OC, AE, etc., at or near their mid-points. The remaining three sets show that atoms occur on those lines at other positions than at the middle point.
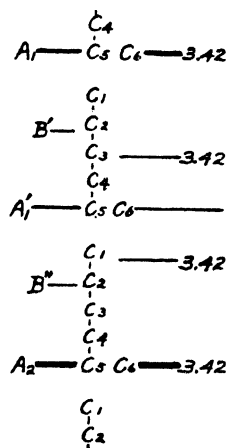
Fig. 4.

In the preceding discussion we have attempted to make clear the steps in an interpretation of the diffraction patterns which led to the conclusion that a lattice arrangement of $C_6H_{10}O_5$ groups exists in the fiber wall. These groups acting as units appeared to be associated as though they were attached to form long parallel chains running lengthwise of the fiber.

Orientation of the C_6 Groups.

It seems probable, from the data now at hand, that the orientation of the C_6 groups might be determined. There is some evidence of a chemical nature that a linkage between these groups exists,[2] and it is thought that carbon 1 is attached through an oxygen atom

to carbon 5 of an adjoining group. In Fig. 4 two C_6 groups are placed on lines corresponding to those of Fig. 2. Each group is represented by the numbered carbons following the conventional method of the chemist only in assigning a number to each carbon atom. No attempt is made here to locate the atoms spatially, except in a most general way. With the groups oriented in this way the A planes would be equal to one another in reflecting power and the B planes would also have equal values in reflection. When considering the 3.42 planes it is immediately seen that A'_1 would annul completely the reflection from A_1, B'' would annul B', etc. The result, of course, would be that no 3.42 line would be formed. The same effect would be produced for all of the odd order lines,—3.42, 2.03, 1.46, 1.14, etc. It seems then that a linkage between carbon 1 and carbon 5 will not satisfy our interpretation of the diffraction pattern.

If the groups are attached to one another the alternative is a linkage between C1-C1 and C5-C5.

In addition to the orientation of the adjacent groups in each chain in that manner, the structure seems to be still further complicated by a difference in orientation of the chains themselves, as represented in Fig. 8. Whether there is justification in revolving alternate chains as much as 180° is perhaps open to question. That arrangement is indicated by the occurrence of alternating light and heavy planes in the (111) series, Table VI, and by the absence of such an arrangement of planes in the (101) and (011) series, Tables IV and V. In Table VI the even numbered planes only, (112), (114), (116), etc. are sets of alternating light and heavy planes, while the odd numbered are sets of like planes. In the other two tables all are sets of like planes.

That situation may be illustrated by the following figures, 5 to 10, in which the horizontal lines represent the transverse planes in the fiber, which are spaced 10.25 Å.u. as A_1 and A_2 in Fig. 2; and in which the vertical lines represent the longitudinal planes, those to which the long axis of the fiber is parallel.

In Fig. 5 the longitudinal planes have a spacing of 3.98 Å.u. The diagonal lines in the figure are edge views of (114) planes. Their spacing is 2.17. On the diffraction patterns there appears a line

which seems to be associated with this same set of planes since it indicates a spacing of double their value; that is, 4.35. If these two lines, 2.17 and 4.35, are produced by the same set of planes, then light and heavy planes must alternate in the set and we may say that the atoms have a *different arrangement* in the heavy planes from that in the light ones. In the diagram, Fig. 6, that condition is represented by light and heavy solid lines. At their intersection with the A_1
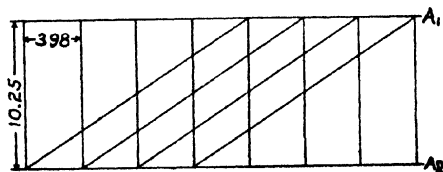
Fig. 5.
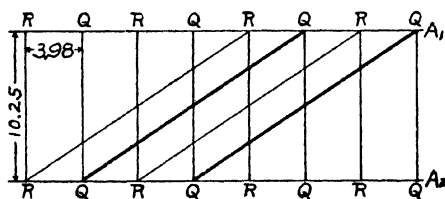
Fig. 6.
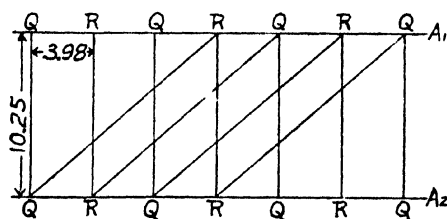
Fig. 7.

transverse plane, two different arrangements of the atoms are assumed to occur alternately, which are designated as Q and R arrangements. Since plane A_2 is identical to A_1 the Q's and R's would be repeated. The result is that the heavy planes have only Q arrangements in them and in the light planes only R arrangements. All of the even numbered sets, (112), (114), (116), etc., will be of this type.

The odd numbered sets, however, will be seen to contain both

Q and R arrangements instead of all of the one or of the other, and thus each plane will have the same reflecting power, in a given set, as any other plane. Fig. 7 represents the (113) planes where each plane is of that type; that is, it contains both Q's and R's.

This QR arrangement of the atom groups seems to be in agreement with the assortment of planes in Table VI and may mean that the difference between the chains of atom groups which extend through RR and those which extend through QQ is only a difference in orientation of the alternate chains; that is, that the adjacent chains are turned laterally on their long axes so that only the alternate ones have the same orientation. In Fig. 8 an attempt is made to picture this arrangement by revolving every other one through 180° as an extreme case.

Such a structure in perspective would appear somewhat as in Fig. 9 where the Q and R conventions are used again to avoid confusion. This might represent a minute section taken out of the wall of a fiber in which QQ and RR are parallel to the long axis of the fiber. The upper face is the A_1 plane of previous figures, the lower is A_2. The diagonal planes shown in the figure contain at the intersections with A_1 and A_2 either all Q or all R arrangements. Thus Fig. 5, Fig. 6, and Fig. 7 would each represent a view of this perspective as though one looked along MN. The corner COA has the same lettering as in Fig. 3 so that one may compare the planes of the two figures.

A structure built to accord with the planes of the (111) series should also be in agreement with those of the (101) and (011) series. In the latter all planes of a given set are equal in reflecting power and therefore no double spacing occurs. That must mean that every plane has both Q and R arrangements in it. When we make a two dimensional view of the perspective Fig. 9 as though looking along MP or MO, we see that every plane contains both Q's and R's, as in Fig. 10, so that in any set of these diagonals all planes are equal in reflecting power, as called for in the tables. Apparently, then, the structure is in agreement with the diagonal planes as determined from the photographs taken at various positions of the fiber block.

Further consideration of the structure shows that the transverse planes of Table I would not be affected by this rotation of alternate chains. There might be, however, a question as to the effect on the
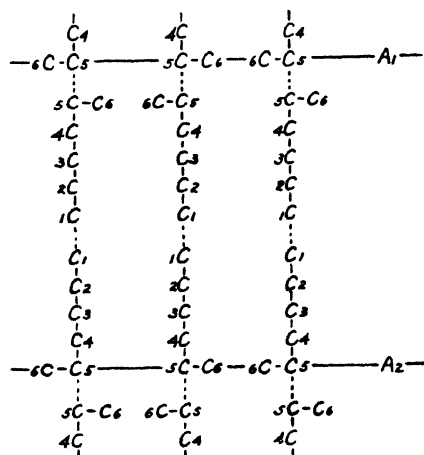
FIG. 8.
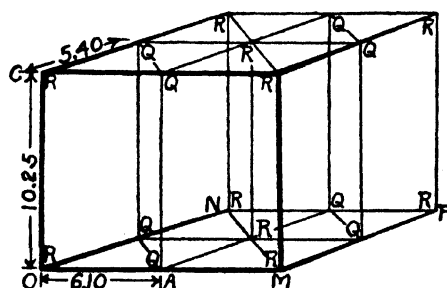
FIG. 9.
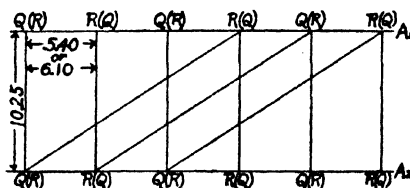
FIG. 10.

longitudinal planes of Table VII where the (110) and (130) alter-
nate planes would contain either all R or all Q orientations. In spite
of the apparent difference between the adjacent planes of these sets,

it is highly probable that they have equal, or very nearly equal, reflecting powers since the atoms seem to be so closely grouped around the central axis of the chain.   In other words, the patterns from the 0° position of the fiber block show that few if any of the heavier atoms occur between the planes of the three prominent sets, (100), (010), and (110) as discussed above, and therefore the close arrangement of the atoms would give each chain practically the same power as every other chain no matter how turned on its long axis, resulting in adjacent planes having practically equal reflecting powers.

SUMMARY.

It has been shown that the wall of the plant fiber is probably built up of unit groups of atoms which have assumed the form of a space lattice.   The elementary cell of the lattice is an orthorhombic structure with the dimensions $6.10 \times 5.40 \times 10.30$ Å.u., and contains two unit groups equal in size to two $C_6H_{10}O_5$ groups.   The crystallographic unit cell would contain 4 of these elementary cells and would be represented by Fig. 9 rather than by Fig. 3.

The groups of atoms, $C_6H_{10}O_5$, are arranged in parallel chains running lengthwise of the fiber.   In each chain the odd numbered *groups* have a different orientation from the even numbered.   The chains, parallel to one another are spaced 6.10 Å.u. in one direction and 5.40 Å.u. at right angles to that.   In these two directions the odd numbered *chains* also would have a different orientation from the even numbered.

On account of the cylindrical shape of the fiber, the elementary cells are arranged in the form of concentric cylinders or layers. The dimensions of the fibers are such that the fiber wall is about 40,000 elementary cells in thickness, or in other words, the fiber is composed of that many concentric layers.   If it could be magnified sufficiently, a cross-section of a fiber would show the end view of each cylinder as a dotted circle.   The dots, representing the unit groups of atoms, would have considerable uniformity of spacing in both the tangential and the radial directions, 6.10 Å.u. in one and 5.40 Å.u. in the other.   The structure could not be as rigidly exact as might be inferred, since the wall is deposited more or less rhyth-

mically during a period of several days or weeks[5] in which adjustments in the arrangement of the unit groups undoubtedly occur. It is common knowledge that the fibers, under the microscope, rarely appear as true circles on cross-section; usually they appear as irregular, many-sided polygons and the wall thickness is normally uneven. For our purpose it is simpler to think of the fiber as composed of concentric cylinders with diameters so large in proportion to the size of the unit groups that in relatively large segments they closely approach the parallelism of the planes of a rectangular lattice, sufficiently close to be capable of producing diffraction patterns.

Although these conclusions seem to be in agreement with the diffraction patterns obtained from various positions of a bundle of approximately parallel fibers, the fact must not be overlooked that the structure cannot be proved with as great certainty as can the structure of a well formed crystal. The very nature of the fiber, its cylindrical shape, and the many internal adjustments which must take place, militate against a clean-cut demonstration.

Models, made more or less to scale, were used in working out this structure. The unit group was constructed according to Irvine's suggestion[2] that all the groups are glucose residues. An intensive study is now under way in which an attempt is being made to bring the models into agreement with the chemical and physical properties of the cellulose fibers and with the diffraction patterns. A report on that part of the work will soon be submitted for publication.

[5] Balls, W. L., The development and properties of raw cotton, London, 1915, 73; also *Proc. Roy. Soc. London, Series B*, 1919, xc, 543.

THE KINETICS OF OSMOTIC SWELLING IN LIVING CELLS.

BY MORTON McCUTCHEON AND BALDWIN LUCKE.

(From the Laboratory of Pathology, School of Medicine, University of Pennsylvania, Philadelphia, and the Marine Biological Laboratory, Woods Hole.)

The present paper is the first of a series of studies on volume changes in living and dead cells. The simplest case of such volume change, and therefore the easiest and most satisfactory to investigate quantitatively, is the swelling of living cells in hypotonic solutions.

This study is concerned with the *rate* of swelling, and with the way in which the rate is affected by temperature and by the osmotic pressure of the outside solution. The material selected was the unfertilized egg of the sea urchin, *Arbacia.*

This cell is ordinarily in osmotic equilibrium with sea water, which is its natural medium. If sea water is diluted with distilled water, there occurs diffusion of water into the cell which swells, until it again reaches osmotic equilibrium with the outside solution.

For the study of osmosis, the sea urchin egg is particularly favorable material. When the egg is placed in a hypotonic solution and observed under the microscope, the diameter of the egg increases in a very regular way. And since the egg is spherical, the change in volume may easily be calculated from the change in diameter. If measurements are made at minute intervals with an ocular micrometer, and plotted against time, a curve is obtained such as is shown in Fig. 1.

Thus we have an admirable means of studying the *velocity* of osmotic swelling. Most studies of osmosis and of osmotic pressure have been concerned only with the state of equilibrium, or with the amount of swelling or of plasmolysis at equilibrium. A few studies have been made on the initial rate of flow. But in the sea urchin egg the process can be followed practically from beginning to end.

Method.

Eggs from a single animal were used in each of the 7 groups of experiments. Their average size was found by measuring 10 to 30 cells. For this purpose a screw ocular micrometer and a 10 mm. objective (immersed in the sea water) were used, the system giving a magnification of 240 diameters.

The eggs were concentrated by slight centrifugation, and a few were then placed in about 50 cc. of hypotonic sea water at known temperature. This solution was contained in a glass dish; its temperature was regulated by a surrounding jacket of water within a larger glass vessel which rested on the stage of a microscope. A thermometer was placed so that the bulb was immersed in the solution containing eggs and in immediate proximity to them. With this arrangement variations in temperature of the solution did not usually exceed ± 0.2°C.

Except for a very small percentage of slightly ovate eggs, which were never used for measurement in these experiments, the eggs of *Arbacia* are spherical.

This fact was repeatedly demonstrated by rolling them over with a fine glass needle, a procedure which readily demonstrates any flattening of the egg, such as occurs when they are placed in boiling water. No such flattening was found before or during osmotic swelling. The spherical shape is preserved unless cytolysis occurs; after this event measurement is impossible. But until cytolysis occurred, the eggs were in general uninjured by the experimental procedure, since, when they were returned to ordinary sea water and sperm added, cleavage usually resulted.

The Rate of Swelling.

Since the process of osmotic swelling is primarily one of diffusion it is to be expected that when the cell is far from osmotic equilibrium with its surroundings it will swell rapidly, but that the rate of swelling will steadily decrease as equilibrium is approached. The same course is followed by unimolecular chemical reactions. The general equation for such a process is $\frac{dx}{dt} = k\,(a\text{-}x)$, where $\frac{dx}{dt}$ is the rate of transforma-

tion at time t, a is the original amount of substance, x is the amount of substance converted up to a time t, and k the velocity constant.

This is a familiar equation since it expresses a variety of processes besides chemical reactions of the first order, such as rate of diffusion, rate of cooling of bodies, and rate with which vibrations of a spring decrease in amplitude. The mistake must not be made of regarding all processes which follow this equation as being unimolecular reactions.
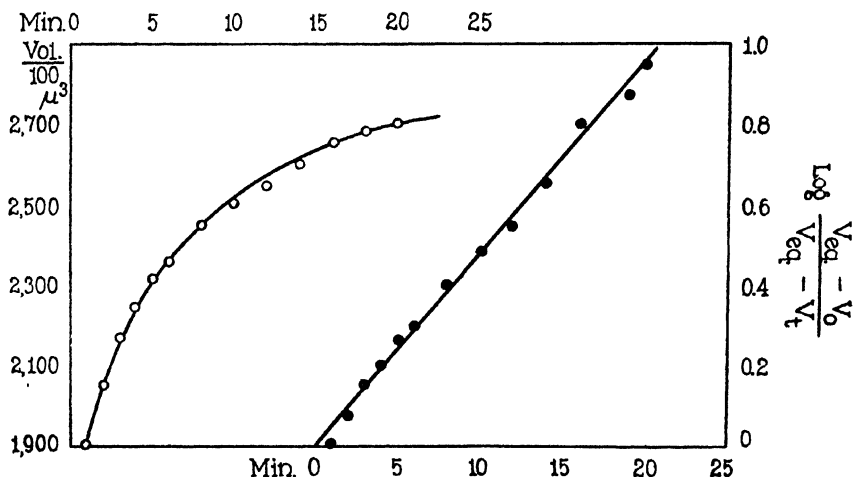
FIG. 1. The rate of swelling of unfertilized sea urchin eggs in 60 per cent sea water at 24.8°. On the left side, volumes are plotted against times. On the right side $\log \dfrac{V_{eq.} - V_0}{V_{eq.} - V_t}$ is plotted against time.

$V_{eq.} = 2707 \times 10^2 \ \mu^3$, $V_0 = 1909 \times 10^2 \ \mu^3$, and the values of V_t are the same as used in the curve on the left. The points fall along a straight line, the slope of which gives the value of k ($= 0.048$). This graph shows the process follows the equation $kt = \ln \dfrac{V_{eq.} - V_0}{V_{eq.} - V_t}$.

Integrating, we obtain $kt = \ln \dfrac{a}{a-x}$. A modification of this suggested by R. S. Lillie (1) as convenient for calculation of volume changes in cells, is, $kt = \ln \dfrac{V_{eq.} - V_0}{V_{eq.} - V_t}$ where $V_{eq.}$ is volume at equilibrium. V_0 is volume at the first instant, and V_t is volume at time t.

It was found by Lillie that this equation correctly represents the rate of swelling of fertilized and unfertilized *Arbacia* eggs in 40 per cent sea water. That it also fits our data may be seen from Fig. 1. When $\log \dfrac{V_{eq.} - V_0}{V_{eq.} - V_t}$ is plotted against time, a straight line should be obtained, and the slope of this line gives the value of k, the velocity constant. It is observed that the points do actually fall on a straight line, within the limits of experimental error. In Fig. 2, it is seen that the equation holds over a wide range of temperatures, and, in Fig. 4, over a wide range of osmotic pressures.

It can therefore be safely concluded that the equation $kt = ln$ $\dfrac{V_{eq.} - V_0}{V_{eq.} - V_t}$ does correctly express the swelling of cells in hypotonic sea water, as would be expected in a diffusion process.

The Effect of Temperature.

We now consider evidence that other factors in addition to diffusion are involved in the rate of swelling. We will consider first the effect of temperature.

A pure diffusion process is only moderately accelerated by increasing the temperature. For a rise of 10°, the rate of such a process would ordinarily be increased about 20 to 30 per cent; in other words the temperature coefficient, Q_{10}, would equal about 1.2 to 1.3. But in our experiments, the effect of temperature was much greater than this.

A typical experiment (one of seven) is represented in Table I. In the first column are placed the times in minutes, in the second, the volumes at 11°C., in the third, at 13.1°, and so on for a series of temperatures. Each volume represents the mean of 3 to 6 cells. The original volume was determined by averaging 10 or 20 cells at the beginning of the experiment, and the volume at equilibrium was observed or calculated.[1] The volume at equilibrium was apparently

[1] $\dfrac{V_0}{V_{eq.}} = \dfrac{\text{intracellular osmolar concentration at equilibrium}}{\text{intracellular osmolar concentration at first instant}}$.
Thus, for experiments in 40 per cent sea water the calculation would be $\dfrac{100}{100 + x}$ $= \dfrac{40}{100}$. Equilibrium volumes were measured directly in several experiments by keeping the cells at low temperature until no further increase in volume occurred. In 80 and 60 per cent sea water the observed volume at equilibrium was 3 per cent less than the calculated; in 40 per cent sea water, 10 per cent less.

independent of the temperature. In the bottom row are given the velocity constants for the different temperatures.

It is seen that the value of k increases rapidly with the temperature, from 0.011 at 11° to 0.024 at 20.5°. The effect of temperature is shown graphically in Fig. 2, where $\log \dfrac{V_{eq.} - V_0}{V_{eq.} - V_t}$ is plotted against time. The slope of the lines gives values of k at various temperatures.

TABLE I.

A Typical Experiment on Swelling of Unfertilized Arbacia Eggs in 40 Per Cent Sea Water. Volumes $\times 10^{-2}$ Are Given in Cubic Micra. Each Number Represents the Mean Volume of 3 Cells. In the Bottom Row Is Given the Velocity Constant for Each Temperature.

Time.	11.0°C.	13.1°C.	14.8°C.	17.6°C.	20.5°C.	22.8°C.	24.8°C.
min.							
1	2310	2330	2180	2437	2310	2340	2365
2	2388	2355	2330	2581	2460	2515	2580
3	2482	2438	2400	2665	2640	2717	2730
4	2518	2615	2496	2767	2782	2835	2975
5	2580	2645	2570	2852	2905	2978	
6	2645	2745	2665	2980	2980	3195	3240
8	2768	2850	2770	3120	3145	3465	3510
10	2875	3010	2880	3370	3390	3736	3815
12	2993	3105	3004	3540	3615	3940	4105
14	3105	3290	3120	3758	3817	4140	4210
16	3215	3370	3215	3836	3920		
18	3271	3540	3346		4091		
20		3585					
$k =$	0.011	0.014	0.012	0.021	0.024	0.033	0.036

Over the range studied the value of the temperature coefficient is fairly uniform at about 2.4, that is, the velocity constant would be increased 2.4 times by a rise in temperature of 10°. In other experiments Q_{10} varied between 2 and 3, but was always fairly uniform throughout the temperature range in any given experiment (see Table II).

This magnitude of the temperature coefficient is a common one in chemical reactions. But in pure diffusion processes, the temperature coefficient is usually low, about 1.2 or 1.3.

In the last 2 years another measure for the effect of temperature on biological processes has been strongly advocated by Crozier and others (2). It was found by Arrhenius that in certain chemical reactions the velocity is proportional to the reciprocal of the absolute temperature, or, $ln \dfrac{k_1}{k_2} = \dfrac{q}{R}\left(\dfrac{1}{T_2} - \dfrac{1}{T_1}\right)$ where k_1 and k_2 are velocity constants at different temperatures, q is heat of activation, R is the gas constant, and T the absolute temperature.
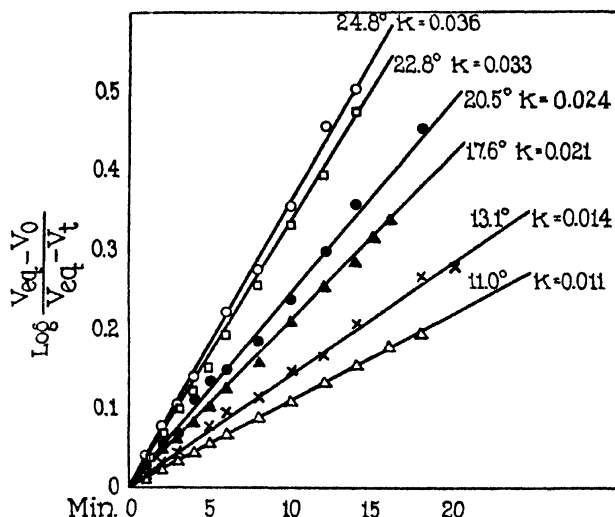
FIG. 2. The effect of temperature on the rate of swelling of unfertilized *Arbacia* eggs in 40 per cent sea water. $\text{Log}\ \dfrac{V_{eq.} - V_0}{V_{eq.} - V_t}$ is plotted against time. The resulting graph is for each temperature a straight line, the slope of which gives the value of k, the velocity constant. The experiment at 14.8° has been omitted to avoid confusion. Data are taken from Table I.

For chemical reactions the value of q usually lies between 10,000 and 30,000 calories, while, if calculated for diffusion processes, its value would be about 7,000.

In Fig. 3, the logarithms of the velocity constants have been plotted against the reciprocals of the absolute temperature. This graph is a composite of three experiments in 40 per cent sea water. The value of q or μ, as it is often called, is about 16,000—corresponding to that of a chemical reaction.

TABLE II.

Summary of Seven Experiments. The Table Shows That the Velocity Constant, k, Varies Directly with the Temperature, and Inversely with the Concentration of the Sea Water. In the Bottom Line Are Given the Values of μ for the Several Experiments.

Temperature °C.	k	Sea water 60 Distilled water 40 Temperature °C.	k	Temperature °C.	k	Sea water 40 Distilled water 60 Temperature °C.	k	Temperature °C.	k	Temperature °C.	k	Sea water 20 Distilled water 80 Temperature °C.	k
9.9	0.016	15.3	0.041	11.8	0.037	11.0	0.011	10.2	0.011	11.7	0.015	10.4	0.004
12.3	0.017	18.8	0.055	14.6	0.071	13.1	0.014	13.5	0.015	12.9	0.013	11.5	0.005
13.2	0.020	24.5	0.074	16.8	0.047	14.8	0.012	15.3	0.017	15.2	0.015	14.8	0.006
15.2	0.023	28.4	0.158	21.3	0.089	17.6	0.021	17.9	0.016	17.7	0.020	17.1	0.007
16.2	0.027	29.3	0.140	22.3	0.085	20.5	0.024	21.9	0.037	20.9	0.034	18.8	0.008
18.5	0.029	32.8	0.190	24.8	0.124	22.8	0.033	23.9	0.034	24.3	0.044	21.2	0.013
18.8	0.024			26.8	0.101	24.8	0.036	26.1	0.045	25.9	0.057		
19.6	0.041			29.8	0.157								
21.8	0.043												
26.3	0.069												
28.3	0.061												
μ....14,500		15,400		14,800		15,600		16,500		19,000		13,400	

Similar results were obtained in three experiments in 60 per cent sea water and in one experiment in 20 per cent sea water. The value of μ in these several experiments ranged from 13,000 to 19,000, but was fairly uniform throughout the temperature range in any one experiment.

It may be concluded from these data that the temperature coefficient of osmotic swelling in living cells is regulated by factors other than diffusion. That the effect of temperature lies in alteration in the permeability of the cell membrane to water seems probable, but at present cannot be proved.
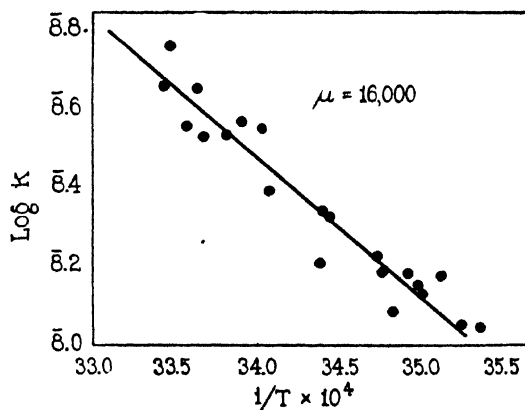
Fig. 3. Swelling of unfertilized *Arbacia* eggs in 40 per cent sea water. The logarithms of the velocity constants of three experiments are plotted against the reciprocals of the absolute temperature. The slope of the line gives the value of μ (= 16,000).

The rate of water absorption in its relation to temperature has hitherto been studied chiefly with plant tissue, either by the method of plasmolysis or by weighing the material. High temperature coefficients have been reported by Krabbe (3), van Rysselberghe (4), Brown (5), and Delf (6). With animal material the most significant work is that of Bialaszewicz (7), who found that unfertilized frog eggs took up water five times more rapidly at 20° than at 10°. But, as Kanitz (8) points out, his method is open to the objection that he compared the amounts of water taken up in equal times instead of the times required to produce a certain fraction of the total amount of swelling.

The literature on the effect of temperature on absorption of water has been reviewed by Kanitz (8) and Stiles (9). Przibram has lately collected in tabular form all available data of biologic interest on temperature coefficients (10).

While in the light of present knowledge it seems likely that the effect of temperature is exerted chiefly on permeability of the cell membrane, yet it is possible that the effect is a more complicated one. Thus it might be suggested that changes in viscosity possibly play some part in establishing a high temperature coefficient. Available evidence on this point, though not conclusive is against such a supposition. Heilbrunn (11) has studied the effect of temperature on the viscosity of several marine eggs. With *Cumingia* eggs he found that the curve of viscosity passes through a maximum at about 15°. As the temperature rises above 15° or falls below it, the protoplasm becomes more and more fluid until suddenly it undergoes a sharp increase in viscosity, at about 4° and 30° respectively. In the *Arbacia* egg the general relation of viscosity to temperature is similar.

If the temperature coefficient of osmotic swelling were largely influenced by viscosity changes in the cell, we should expect different values of Q_{10} in different parts of the temperature range. This, however, is not the case for, as has been stated above, the value of the temperature coefficient remains practically constant over the range studied, from 10° to 30°. It therefore seems unlikely that viscosity greatly influences the temperature coefficient of osmotic swelling in living cells.

Effect of the Osmotic Pressure of the Solution.

If one egg is placed in a mixture of sea water and distilled water containing 60 per cent sea water, and another egg is placed in 40 per cent sea water, it is evident that the latter egg will take up water more rapidly and swell to a greater volume than the former. But it is not evident which egg will reach equilibrium first (or, more correctly, swell half way to equilibrium first).

The answer to this question can be obtained from the data already given (Table II). As seen in Fig. 4, it took much longer for cells to swell in 20 per cent sea water than in 40, and longer in 40 per cent than in 60 per cent. The velocity constants become correspondingly lower

as the concentration of sea water is decreased.   In the typical example given in the chart, at $15 \pm 0.2°$ for 80 per cent sea water, $k = 0.072$, for 60 per cent sea water, $k = 0.024$, for 40 per cent, 0.012, and for 20 per cent, 0.006.
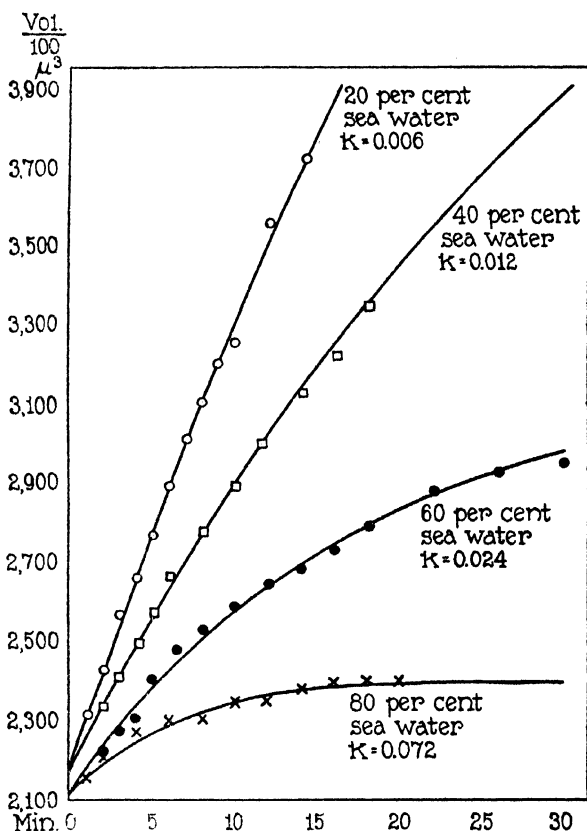
FIG. 4. The effect of osmotic pressure of the solution on the rate of swelling of unfertilized *Arbacia* eggs, at $15 \pm 0.2°C$. Volumes are plotted against times. Points represent *observed* volumes; curves represent *calculated* volumes $\left(kt = ln \dfrac{V_{eq.} - V_0}{V_{eq.} - V_i} \right)$.

Our data are not at present adequate for working out the law that connects concentration with rate of swelling, but the general principle stands out clearly, that the more dilute the sea water is made, the longer it takes the cell to swell.

SUMMARY.

The rate of swelling of unfertilized sea urchin eggs in hypotonic sea water was investigated. Analysis of curves leads to the following conclusions.

1. The rate of swelling follows the equation, $kt = ln \dfrac{V_{eq.} - V_0}{V_{eq.} - V_t}$ where $V_{eq.}$, V_0, and V_t stand for volume at equilibrium, at first instant, and at time t, respectively, the other symbols having their usual significance. This equation is found to hold over a wide range of temperatures and osmotic pressures. This relation is the one expected in a diffusion process.

2. The rate of swelling is found to have a high temperature coefficient ($Q_{10} = 2$ to 3, or $\mu = 13,000$ to $19,000$). This deviation from the usual effect of temperature on diffusion processes is thought to be associated with changes in cell permeability to water. The possible influence of changes in viscosity is discussed.

3. The lower the osmotic pressure of the solution, the longer it takes for swelling of the cell. Thus at $15°$ in 80 per cent sea water, the velocity constant has a value of 0.072, in 20 per cent sea water, of 0.006.

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THE EFFECT OF HYDROGEN ION CONCENTRATION ON SWELLING OF CELLS.

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It has been repeatedly stated in the literature that mammalian cells increase in volume when placed in acid solutions. Conversely, it has been thought that the swelling of tissue cells observed in various pathological conditions is often the result of acid production (1). The process has been supposed to be similar to that producing volume changes in such dead systems as gelatin, when the H^+ concentration of the solution is altered.

Erythrocytes are known to swell in acid solutions. This volume change has been shown by Van Slyke and his associates (2) and by Warburg (3) to be dependent on the facts that the membrane of the erythrocyte is permeable to anions, but not to cations or hemoglobin, and that the amount of base in combination with hemoglobin is a function of the H^+ concentration; alteration in the latter produces change in osmotic pressure within the cell, in accordance with the Donnan equilibrium, and hence volume change of the cell.

The mammalian erythrocyte is, however, a highly specialized cell, in respect to function, as well as low metabolism, inability to divide, and absence of nucleus. It is, therefore, not permissible to assume that a mechanism for volume change exists in living nucleated cells such as is present in erythrocytes and dead systems. In fact recent work tends to show that living cells are relatively impermeable to both cations and anions as long as they remain uninjured.

We were, moreover, inclined to question whether swelling of living cells in acid solutions was a well established fact. In previous work it is not clear whether cells were alive or dead at the end of the experiment. But, as will be seen, it is precisely this question of the life or death of the cell that determines whether it will swell in acid solutions.

The problem was, therefore, to determine whether the volume of living cells depends on the pH of the solution, as is the case with erythrocytes. It was necessary to select a type of cell that can be readily measured, that is more comparable, both in structure and function, to tissue cells than is the case with mammalian erythrocytes, and finally a cell of which the viability can be definitely determined. Unfertilized *Arbacia* eggs meet these requirements. In common with tissue cells they possess a nucleus, and the ability to divide. Large numbers of comparable cells can be obtained from a single animal and examined in sea water, their natural environment, hence without injury, except such as is deliberately induced. Since they are spherical, changes in volume can readily be calculated from changes in diameter. At the end of the experiment, viability can be tested by attempting fertilization.

Method.

Solutions were prepared as follows: Sea water was neutralized with HCl and aerated overnight by means of a water pump. HCl or NaOH was then added to obtain the desired range of pH values, from 3.0 to 9.8, measured colorimetrically. Pyrex tubes of 100 cc. capacity were nearly filled with these solutions and stoppered. Eggs were obtained from a single animal for each experiment. They were washed twice in sea water, concentrated by slight centrifugation, washed again in the solution to be tested, and a few placed in each tube, at room temperature. The tubes were frequently agitated. In control tubes were placed identical solutions, eggs, and a few drops of an indicator solution. Changes of pH during an experiment were found to be generally insignificant.

At stated intervals, samples of eggs were removed from the tubes. The diameters of 10 eggs were measured with a 10 mm. objective and an ocular screw micrometer. With this system a magnification of 240 diameters was obtained. The eggs were then returned to ordinary sea water and a freshly diluted suspension of sperm added. The cells were examined for cleavage after about 2 hours.

In a second series of experiments washed CO_2 was bubbled through neutralized sea water, or aqueous NH_4OH added to obtain the desired pH range (4.2 to 9.8). In control tubes, changes in pH during experiments were usually slight.

TABLE I.

Effect of H Ion Concentration (HCl and NaOH) on Volume of Unfertilized Arbacia Eggs.

Control = 75.7 ± 0.4 μ in Diameter.

pH	3.0	4.0	5.0	6.0	7.0	8.0	9.0	9.8
Time. min.								
4	75.2 ± 0.4 C.	75.4 ± 0.3 C.	75.3 ± 0.3 C.	74.9 ± 0.3 C.	75.4 ± 0.4 C.	73.9 ± 0.4 C.	75.5 ± 0.3 C.	75.0 ± 0.4 C.
16	75.9 ± 0.3 A.C.	76.3 ± 0.3 C.	75.2 ± 0.2 C.	75.0 ± 0.4 C.	76.3 ± 0.4 C.	75.3 ± 0.4 C.	75.0 ± 0.3 C.	75.3 ± 0.3 C.
64	76.5 ± 0.6 N.C.	76.2 ± 0.5 N.C.	74.1 ± 0.2 A.C.	74.9 ± 0.3 C.	75.5 ± 0.4 C.	74.6 ± 0.3 C.	74.9 ± 0.3 C.	74.6 ± 0.2 C.
256	83.4 ± 0.5 N.C.	78.8 ± 0.8 N.C.	75.8 ± 0.2 A.C.	74.7 ± 0.3 A.C.	75.4 ± 0.2 C.	75.5 ± 0.2 C.	75.3 ± 0.2 C.	75.8 ± 0.4 C.

Each figure represents the mean diameter in micra, plus or minus its probable error, of 10 cells. After measurement the cells were returned to ordinary sea water and sperm was added; C. = normal cleavage; A.C. = atypical cleavage; N.C. = no cleavage.

Results of Exposure to HCl and to NaOH.

In Table I are given the results of one of three similar experiments. It will be seen that changes in volume occurred only in the two most acid solutions, *i.e.* at pH 3.0 and 4.0, and in these only after long exposure to the acid. An interesting and significant relation is brought out between volume change and the ability of the egg to develop: loss of power to develop precedes volume change. Thus at pH 3.0,

TABLE II.

Effect of H Ion Concentration (CO_2 and NH_4OH) on Diameter of Unfertilized Arbacia Eggs.
Control = 75.7 ± 0.1 μ in Diameter.

pH.........	4.2	6.0	7.0	8.0	9.0	9.8
Time.						
min.						
4	76.0 ± 0.2 N.C.	75.4 ± 0.3 C.	75.4 ± 0.2 C.	77.3 ± 0.3 C.	76.0 ± 0.2 C.	76.2 ± 0.3 C.
16	75.0 ± 0.3 N.C.	75.7 ± 0.2 C.	75.9 ± 0.3 C.	74.4 ± 0.3 C.	75.9 ± 0.3 C.	75.7 ± 0.2 A.C.
64	75.8 ± 0.2 N.C.	75.5 ± 0.2 C.	75.0 ± 0.3 C.	75.6 ± 0.2 C.	76.3 ± 0.3 A.C.	76.4 ± 0.3 A.C.
256	76.9 ± 0.3 N.C.	74.9 ± 0.2 A.C.	74.9 ± 0.3 C.	75.5 ± 0.3 C.	75.5 ± 0.3 N.C.	75.8 ± 0.3 N.C.

Each figure represents the mean diameter in micra, plus or minus its probable error, of 10 cells. After measurement the cells were returned to ordinary sea water and sperm was added. C. = normal cleavage; A.C. = atypical cleavage; N.C. = no cleavage.

after 4 minutes exposure, there was no volume change and normal cleavage followed fertilization. After 16 minutes, there was no change in size, but the eggs subsequently divided atypically. After 64 minutes there was no change in size, but the eggs failed to divide at all. After 256 minutes the eggs showed marked swelling and no cleavage occurred.

At pH 4.0 the changes were less marked, but in the same direction. The fact, therefore, stands out clearly that no swelling of the eggs

occurred in solutions of HCl until *after* the eggs had suffered serious injury: injury so severe that they were incapable of subsequent development. That the eggs at the time of swelling were not only injured, but actually dead, is altogether probable.

Results of Exposure to CO_2 and to NH_3.

The effects produced by these substances were similar to those obtained with HCl and with NaOH. In Table II are given the results of a typical experiment. It is seen that, though there was marked inhibition of subsequent cleavage, there was, with the concentrations of CO_2 and NH_3 used, no change in size as long as the cells remained uninjured.

DISCUSSION.

That the results obtained with *Arbacia* eggs cannot, without experimental evidence, be applied to all cells, is sufficiently evident. Bearing this fact in mind, our conclusions may be stated as follows:

Our experiments tend to show that cells other than erythrocytes do not swell in HCl, NaOH, CO_2, or NH_3 as long as they are alive. That this is true of HCl and NaOH is perhaps not surprising, since recent work appears to show that uninjured cells are relatively impermeable to all ions. But with CO_2 and NH_3 the case is quite different. Here we are dealing with substances known to penetrate cells with great readiness and to alter their hydrogen ion concentration (4). Under these circumstances both erythrocytes and dead proteins such as gelatin would undergo volume change, due to alteration in the amount of protein in combination with acid or base. Possibly no such combination occurs in the case of living protoplasm. However that may be, our experiments appear to show that the volume of living cells, in contrast to that of erythrocytes and dead proteins, is independent of H^+ concentration.

Finally the fact remains that our results differ from those of previous workers who found that cells swelled in acid solutions. Whether this discrepancy depends on differences in material or whether the cells observed by former workers were injured or dead, remains for the present an open question. In general, however, it seems to be true that if the internal reaction of a cell is made acid enough to cause swelling, the cell is seriously injured (Crozier (5)).

SUMMARY.

1. The effect of HCl, NaOH, CO_2, and NH_3 on the volume of un-fertilized *Arbacia* eggs was tested over a wide range of pH values.

2. No swelling occurred, except in HCl solutions, and there not until after injury or death had occurred.

3. Whereas the volume of erythrocytes and of proteins such as gelatin is known to be dependent on the pH of the solution, such a relation does not exist in the case of living and uninjured cells, at least of the type tested.

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AN EMPIRICAL FORMULA FOR THE RELATION BETWEEN VISCOSITY OF SOLUTION AND VOLUME OF SOLUTE.

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It has been established by a number of researches that Einstein's equation for the viscosity of solutions, as derived originally in 1906[1] and then modified in 1911,[2] is not applicable even to moderate concentrations of colloidal solutions. This equation expresses viscosity as a linear function of the volume fraction of the dispersed substance by the formula,

$$\eta = 1 + 2.5\varphi$$

where η is the relative viscosity of the suspension, *i.e.* the ratio of the absolute viscosity of the suspension to that of the pure solvent, while φ is the volume occupied by the dispersed substance expressed as a fraction of the total volume of the solution.

This formula is an approximation of the expression

$$\eta = \frac{1 + 0.5\varphi}{(1 - \varphi)^2}; \tag{1}$$

Examination of Einstein's paper revealed the fact that according to the printed derivation, the complete equation should be

$$\eta = \frac{1 + 0.5\varphi}{(1 - \varphi)^4} \tag{2}$$

instead of $\eta = \dfrac{1 + 0.5\varphi}{(1 - \varphi)^2}$, and it was found, as will be shown below, that formula (2) agrees very closely with the experimental facts. Correspondence with Professor Einstein, however, showed that the

[1] Einstein, A., *Ann. Physik*, 1906, xix, 289.

[2] Einstein, A., *Ann. Physik*, 1911, xxxiv, 591.

apparent error was due to a misprint in that the expression as printed,[3] $\delta = A^2 + B^2 + C^2$, should be $\delta^2 = A^2 + B^2 + C^2$. The equation $\eta = \dfrac{1 + 0.5\varphi}{(1 - \varphi)^4}$ therefore cannot be derived from Einstein's theory but still serves as a useful empirical relation.

On expansion this formula gives

$$\eta = 1 + 4.5\,\varphi + 12\,\varphi^2 + 25\,\varphi^3 \; . \; .$$

When φ is very small, this equation becomes

$$\eta = 1 + 4.5\,\varphi,$$

which is identical with the one derived by Hatschek in 1910[4] for the viscosity of suspensions, by the application of Stokes' formula for a sphere moving through a viscous medium. But it appears from Hatschek's derivation that his formula is not an approximation and hence may be applied to cases where φ is quite large, while the derivation of the formula as given above shows that it is only an approximation and applies only to cases where φ is very small. This becomes especially clear when notice is taken of the rapidly increasing values of the coefficients of the higher powers of φ. It is obvious then that the only proper way for making use of the formula is to employ it in its complete form namely

$$\eta = \frac{1 + 0.5\varphi}{(1 - \varphi)^4}.$$

There is no difficulty in calculating the values of η by means of this formula when the values for φ are given. But, on the other hand, a determination of φ from a given value of η leads to the solution of an equation of the fourth degree. This difficulty can be overcome by plotting the curve shown in Fig. 1, representing the equation $\eta = \dfrac{1 + 0.5\varphi}{(1 - \varphi)^4}$. This was done by assuming various values of φ and solving for η. The values of φ for various values of η may then be read off directly from the curve. This method was used for determining the values of φ for various solutions, as given in Tables I to V. For

[3] Einstein, A., *Ann. Physik*, 1906, xix, 296, 300.

[4] Hatschek, E., *Z. Chem. u. Ind. Kolloide*, 1910, vii, 301.

Relation between relative
viscosity of a solution and
the volume fraction of the
solute from the formula
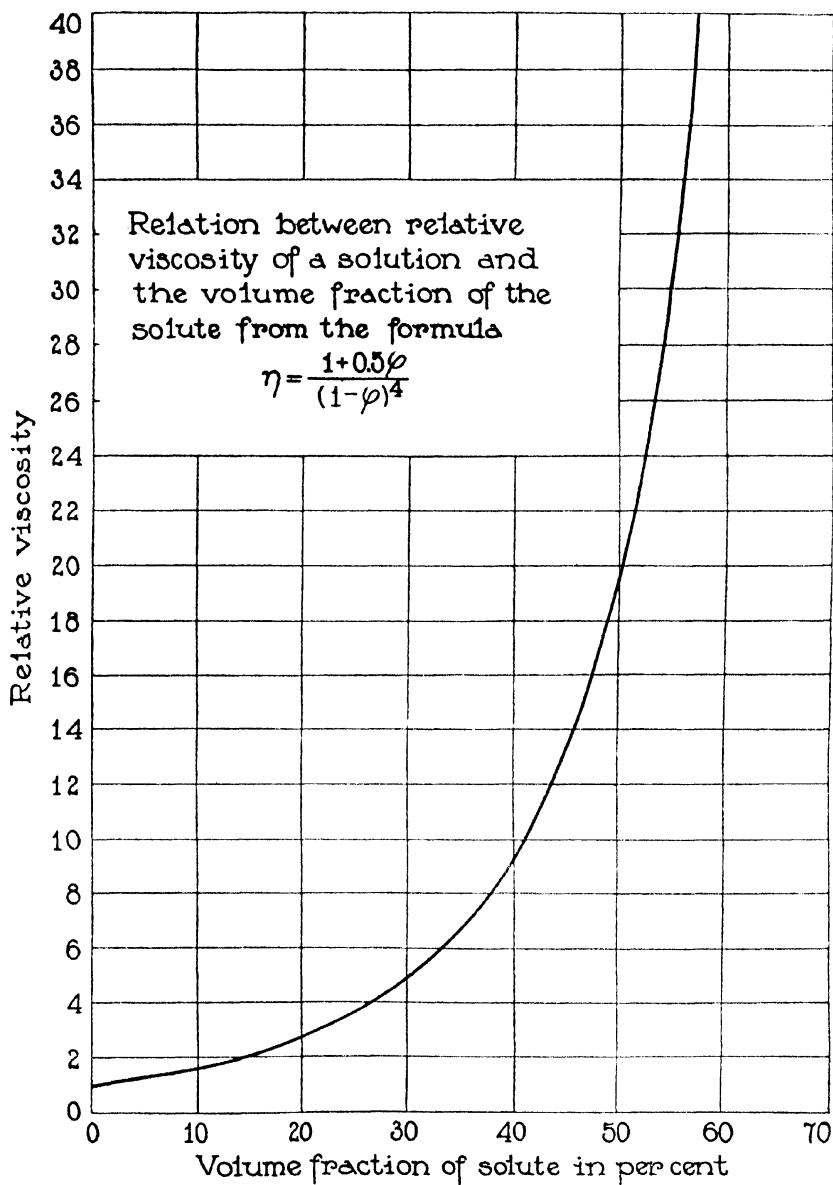
$$\eta = \frac{1 + 0.5\varphi}{(1-\varphi)^4}$$

FIG. 1.

TABLE I.

Viscosity of Solutions of Various Sugars in H_2O at 25°C.
(From the data by Pulvermacher, O., *Z. anorg. u. allg. Chem.*, 1920, cxiii, 147.)

Gm. per 100 cc. solution.	Relative viscosity.	Calc. from formula, $\eta = \dfrac{1 + 0.5\,\varphi}{(1 - \varphi)^4}$.		Specific volume.	
		Volume fraction of solute.	Specific volume.	Calc. from formula, $\eta = 1 + 2.5\,\varphi$.	Observed, Chemists' Year Book, 1925.
		Saccharose.			0.63 (20°C.)
		per cent			
1.0	1.026	0.6	0.60	1.04	
2.0	1.054	1.2	0.60	1.08	
4.9	1.141	3.0	0.61	1.14	
10.3	1.329	6.2	0.60	1.28	
15.6	1.570	9.6	0.61	1.46	
21.7	1.917	13.7	0.63	1.69	
		Glucose.			0.64
2.1	1.062	1.4	0.66		
4.7	1.130	2.8	0.60		
10.6	1.316	6.0	0.57		
16.6	1.619	10.3	0.62		
21.7	1.901	13.5	0.62		
26.4	2.216	16.5	0.63		
		Galactose.			
1.15	1.031	0.7	0.61		
2.31	1.062	1.4	0.61		
4.67	1.125	2 7	0.58		
9.40	1.283	5.5	0.59		
19.60	1.748	11.9	0.61		
		Lactose.			0.66 (4°C.)
2.8	1.086	1.9	0.68		
5.9	1.187	3.8	0.65		
12.1	1.450	8.0	0.66		
18.3	1.779	12.2	0.67		
25.7	2.371	17.8	0.69		

greater precision two curves were used; one covering a range of η from 1.000 to 5.000 with a scale of 10 cm. per unit of η and 1 cm. per 1 per cent in the values of φ, and another one covering a range of η

from 1.00 to 40.0 with a scale of 1 cm. per unit of η and 0.5 cm. per 1 per cent.

As defined above, the symbol φ stands for the volume fraction of the

TABLE II.

Viscosity of Suspensions of Sulfur Particles in Dilute NaCl.
(From the data by Odén, S., *Z. physik. Chem.*, 1912, lxxx, 709.)

Gm. per 100 cc. solution.	Relative viscosity.	Calc. from formula, $\eta = \dfrac{1 + 0.5\,\varphi}{(1 - \varphi)^4}$.	
		Volume fraction of solute.	Specific volume.
Temperature 25°C. Diameter of particles 100μμ.			
		per cent	
1.28	1.035	0.8	0.62
3.84	1.090	2.0	0.52
7.68	1.230	4.6	0.60
15.36	1.510	8.9	0.58
24.14	1.975	14.3	0.59
30.72	2.450	18.4	0.60
48.28	5.000	30.6	0.63
Temperature 20°C. Diameter of particles 100μμ.			
5	1.13	2.8	0.56
10	1.30	5.8	0.58
20	1.75	11.9	0.60
30	2.40	18.0	0.60
40	3.70	25.6	0.63
45	4.60	29.2	0.65
Temperature 20°C. Diameter of particles 10μμ.			
5	1.095	2.1	0.42
10	1.116	4.1	0.41
20	1.495	8.7	0.44
30	1.99	14.5	0.48
40	2.79	20.8	0.52
45	3.19	23.2	0.51

dispersed substance or of solute in case of solution, and can be expressed either as per cent or as cc. per 100 cc. of solution. Hence φ divided by the weight of the solute per 100 cc. of solution stands for the specific volume of the solute and this should be constant for various

concentrations of the dispersions or solutions unless the solute is hydrated or solvated to an extent varying with the concentration. Thus the applicability of the formula $\eta = \dfrac{1 + 0.5\varphi}{(1 - \varphi)^4}$ may be tested for various dispersions by observing whether the calculated values of φ by means of this formula, when divided by the weight of the solute in gm., produce a constant equal to the specific volume of the solute. This was done for various substances, using published data, with the

TABLE III.

Viscosity of Various Hydrosols.

(Cited by Hatschek, E., *Z. Chem. u. Ind. Kolloide*, 1912, xi, 286.)

Gm. per 100 cc. solution.	Relative viscosity.	Calc. from formula, $\eta = \dfrac{1 + 0.5\,\varphi}{(1 - \varphi)^4}$.		Specific volume. (Hatschek.)
		Volume fraction of solute.	Specific volume.	
Glycogen sol. Data by F. Bottazzi and G. d'Errico.				
		per cent		
20	3.548	24.6	1.23	1.86
25	4.548	29.0	1.16	1.90
30	7.370	36.5	1.22	2.18
35	12.22	43.8	1.25	2.21
40	20.55	50.4	1.25	2.15
Casein sol. Data by H. Chick and C. J. Martin.				
4.35	3.37	24.0	5.5	8.04
6.05	6.12	33.5	5.5	9.64
7.06	8.48	38.6	5.5	9.72
8.49	13.66	45.3	5.3	9.37
9.39	23.72	52.1	5.5	9.35

following results.   Table I gives the calculations of φ and of $\dfrac{\varphi}{C}$ ($C =$ gm. of solute per 100 cc. of solution) for various sugar solutions.   The values of $\dfrac{\varphi}{C}$ calculated by means of the formula $\eta = \dfrac{1 + 0.5\varphi}{(1 - \varphi)^4}$ are quite constant and agree with the observed values of the specific volume of the various sugars, as published in the Chemists' Year Book, 1925.   On the other hand, the values of $\dfrac{\varphi}{C}$ for saccharose, as

calculated by means of the Einstein formula, $\eta = 1 + 2.5\ \varphi$, are not constant but continuously increase with the concentration of the solution and give values of 1.04 to 1.69 which differ widely from the observed value of 0.63. Einstein[5] gives the value for the specific volume of saccharose as 0.61 which is still closer to the values as calculated by the complete formula.

Table II shows the same results in case of suspensions of sulfur

TABLE IV.

Viscosity of Solutions of India Rubber in Various Solvents.
(From the data by Kirchhof, F., *Kolloid-Z.*, 1914, xv, 30.)

Solvent.	Gm. per 100 cc. solution.	Relative viscosity.	Calc. from formula, $\eta = \dfrac{1 + 0.5\ \varphi}{(1 - \varphi)^4}$.		Specific volume. (Hatschek.)
			Volume fraction of solute.	Specific volume.	
			per cent		
Benzin........................	0.5	1.9	13.5	27.0	29.2
"	1.0	4.3	28.0	28.0	46.0
"	3.0	94.0	65.5	21.8	33.7
Benzene........................	0.5	2.1	15.6	31.2	33.0
"	1.0	4.7	29.6	29.6	51.5
"	2.0	23.5	52.0	26.0	45.5
"	3.0	97.3	65.8	21.9	34.0
Carbon tetrachloride............	0.5	2.6	19.6	39.2	49.0
" "	1.0	7.5	36.8	36.8	70.0
Tetrachlorethane................	0.5	2.5	18.8	37.6	44.0
"	1.0	6.9	35.5	35.5	63.0

particles. The values for specific volume, as calculated by the formula $\eta = \dfrac{1 + 0.5\varphi}{(1 - \varphi)^4}$, are quite constant, especially in the series at 25°C. and are quite close to the actual specific volume of sulfur which is about 0.5. In the series at 20°C. the value of $\dfrac{\varphi}{C}$ increases slightly with the increase in concentration of the sulfur, but, as is mentioned by Sven Odén, coagulation of the dispersion begins at 20°C. This coagulation

[5] Einstein, A., *Ann. Physik*, 1906, xix, 301.

increases with the concentration, and hence an increase in the volume of the dispersed substance is to be expected owing to the occlusion of water in the clusters of the partially coagulated particles.

Table III shows how well the formula applies to colloidal solutions, such as hydrosols of glycogen and casein. In this table are also given the values of $\frac{\varphi}{C}$ as calculated by Hatschek by means of his formula, $\varphi = \left(\frac{\eta - 1}{\eta}\right)^3$ as developed in 1911[6] for high concentrations of emulsions. A comparison of the two last columns shows the remarkable constancy of the values for the specific volume of the solutes when calculated by the formula $\eta = \frac{1 + 0.5\varphi}{(1 - \varphi)^4}$ as compared with Hatschek's values.

The difference between these two formulæ is also shown in Table IV where the specific volume of India rubber when dissolved in various solvents is calculated from the relative viscosities of the solutions. Again the values of $\frac{\varphi}{C}$ are quite constant when calculated by the formula $\eta = \frac{1 + 0.5\varphi}{(1 - \varphi)^4}$, while Hatschek's last formula does not fit this case at all. Thus it is evident from all the data given that formula (2) may well be applied to practically all cases of solutions or suspensions of particles where the particles are large in comparison with the molecules of the solvent, and to concentrations of the solute as high as to occupy even 50 per cent of the total volume. The values for the specific volume of the solutes as calculated by this formula are not only constant for various concentrations but also agree in the case of the sugar solutions and the sulfur suspension with the actual specific volume of the solutes in the dry state. On the other hand, the values for the specific volume of such "solutes" as glycogen, casein, and rubber, as obtained from the viscosity measurements, are much higher than the actual values for the specific volume of these substances in the dry state, thus showing that the particles of these substances contain some of the solvent. The viscosity measurements supply a means, then, through the application of the formula, for determining the actual volume of the solvent associated with the solute.

[6] Hatschek, E., *Z. Chem. u. Ind. Kolloide*, 1911, viii, 34.

This figure can be obtained by subtracting the volume occupied by the solute in the dry state from the value of φ as calculated from the viscosity of the solution.

An independent method for testing the significance of the values for φ obtained from viscosity measurements is afforded by osmotic pressure measurements. The equation for osmotic pressure of moderate molal concentrations of solutions of substances of high molecular weight may be expressed as

$$P = \frac{RT}{V_0} \cdot \frac{N_1}{N_0} \text{ [7]}$$

where

    V_0 = mol volume of solvent.
    N_0 = number of mols of solvent.
    N_1 = number of mols of solute.

Let

    C = gm. of solute per 100 cc. of solution.
    M_1 = mol weight of solute.
    φ = volume of solute in cc. per 100 cc. of solution.

Then the above equation may be also written as

$$P = \frac{RT}{V_0} \cdot \frac{\dfrac{C}{M_1}}{\dfrac{100 - \varphi}{V_0}} = \frac{RT}{M_1} \cdot \frac{C}{100 - \varphi} = K \frac{C}{100 - \varphi}$$

or

$$K = P\left(\frac{100 - \varphi}{C}\right)$$

Hence, if the values for φ, as calculated from viscosity measurements are real, then, when they are substituted in the last equation, the value of K should be constant for all concentrations of solutions of the same substance. That this is the case is shown in Table V, where data are given for osmotic pressure and viscosity of solutions of various concentrations of caoutchouc in benzene, as determined by W. A. Caspari in 1914. The values for K are practically constant in every

[7] Eucken, A., Fundamentals of physical chemistry, New York and London, 1925, 212.

TABLE V.

Relation between Osmotic Pressure and Viscosity of Caoutchouc Solutions in Benzene at 20°C.

(From the data by Caspari, W. A., *J. Chem. Soc.*, 1914, cv, 2139.)

Gm. per 100 cc. solution.	Osmotic pressure.	Relative viscosity.	Calc. from formula, $\eta = \dfrac{1 + 0.5\,\varphi}{(1 - \varphi)^4}$.		K^*
			Volume fraction of solute.	Specific volume.	
			Partly deviscified caoutchouc.		
	atmospheres		*per cent*		
4.45	0.0235	45.4	59	13.3	0.216
3.37	0.0158	23.6	52	15.4	0.224
2.70	0.0116	16.8	48	17.8	0.224
2.17	0.0088	12.5	44	20.3	0.227
1.04	0.0036	5.1	31	29.8	0.239
			Highly deviscified caoutchouc.		
9.95	0.0487	16.9	48	4.8	0.255
6.89	0.0282	8.8	39	5.7	0.249
4.11	0.0141	4.0	27	6.5	0.250
2.06	0.0060	2.1	15	7.5	0.248
			Gutta percha.		
6.03	0.0500	15.9	47.2	7.8	0.44
3.04	0.0238	4.76	29.5	9.7	0.55
2.04	0.0146	2.65	20	9.8	0.57
1.26	0.0086	2.06	15	11.9	0.58

$$*K = P\left(\frac{100 - \varphi}{C}\right) = \frac{RT}{M_1}.$$

case,[8] thus showing the close relation between viscosity and osmotic pressure, both being functions of the volume fractions of the solute.

[8] It is interesting to observe that in the case of solutions of substances that swell enormously when brought in contact with suitable solvents, as in the case of rubber in contact with various organic liquids, the values of $\dfrac{\varphi}{C}$ as calculated by means of the formula continuously decrease with increase in concentration, as shown in Table V. Nevertheless the independent method of checking the values of φ by means of osmotic pressure measurements shows that the viscosity formula applies even to cases where $\dfrac{\varphi}{C}$ is not constant.

SUMMARY.

It has been found that the expression $\eta = \dfrac{1 + 0.5\varphi}{(1 - \varphi)^4}$ represents very closely the relation between the volume of the solute and the viscosity of the solution. The formula has been applied to a number of experimental results and found to hold very well for as high concentrations as 50 per cent solutions of such substances as sugars, glycogen, casein, and rubber. In the case of various sugar solutions, and also in the case of sulfur suspensions, the volume of the solute as calculated from the viscosity values agrees with the actual volume of the substance in dry state, as determined from specific gravity measurement, while in the case of caoutchouc solutions in benzene the values of φ as calculated from the viscosity measurements fit remarkably well in the equation for osmotic pressure.

RELATIVE SUSCEPTIBILITY TO ARSENIC IN SUCCESSIVE INSTARS OF THE SILKWORM.*

By F. L. CAMPBELL.

(From the Entomological Laboratory of the Bussey Institution, Harvard University, Boston.)

I.

The susceptibility[1] of mammals to poisons at successive intervals during their postembryonic development has been studied in several cases. For example, Schwartze (6) showed that the susceptibility of white rats to strychnine increases from birth to the time of emergence from the "crawling stage", and then decreases up to the time of maturity. The most recent work is that of Takahashi (7), who found that during growth rabbits become more susceptible to some organic compounds, and to others less. Since the age-susceptibility relation does not seem to have been investigated quantitatively in insects, it was thought that a comparison of the susceptibilities[2] of the last four instars of the silkworm to arsenic would be of general interest as a contribution to comparative toxicology and to the analysis of growth. A comprehensive measure of relative susceptibility and of relative toxicity is also proposed in this paper.

II.

Silkworms to be poisoned were selected in the mid-period (2nd or 3rd day) of each instar from two groups of larvæ, each of which had hatched within a period of 48 hours. The members of the first group were used for experiments with pentava-

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* Contributions from the Entomological Laboratory of the Bussey Institution, Harvard University. No. 261.

[1] "Susceptibility" is preferred by the writer to the reciprocal term "resistance", which has electrical implications.

[2] By "comparison of susceptibilities" is understood the comparison of the effects of equal doses per unit weight of animal, or the comparison of doses per unit weight of animal which produce equal effects.

lent arsenic, and those of the second group, which hatched over a month later, for experiments with trivalent arsenic. The two forms of arsenic were fed to the silkworms in neutral aqueous solutions made by titrating the respective acids with NaOH. The methods of measuring and feeding the solutions to full grown larvæ, and of determining survival times, have already been described in detail (2). Larvæ of the earlier instars required only one or, at the most, two drops from the micro burette, and were fed under a binocular microscope. A drop, as deposited on the mouth-parts, covered the mandibles and labrum, and was supported like a gem in its setting by the antennæ and maxillary palpi. It was not imbibed continuously, but by rhythmical pulsations, probably due to the action of the pumping organ in the pharynx described by Verson (8). The same volume of solution per gm. of larva (5 c.mm.) was fed to each silkworm. If this rule had not been followed, it would have been possible to measure and feed larger volumes per gm. to individuals of the first instar weighing about 0.005 gm. The life end-point, indicated by the failure of the prolegs to retract on tactile stimulation of the proleg hairs, was determined for the smaller larvæ under a binocular microscope without removing the insects from the air bath, which was always maintained at a temperature of 27.00 $\pm$ 0.05°C.

The speed of toxic action of doses ranging from 0.025 to 0.200 mg. As^V per gm. is shown in Fig. 1, and of doses between 0.010 and 0.050 mg. As^{III} per gm. in Fig. 2. Lower doses from which larvæ completely recovered were also used, and helped to indicate the probable values of the minimum lethal doses (M.L.D.). Doses higher than 0.050 mg. As^{III} per gm. could not be employed at 5 c.mm. per gm., because of the relatively low solubility of As_2O_3.

III.

Since speed of toxic action may be taken as an "effect" measure of susceptibility,[2] Figs. 1 and 2 show that susceptibility decreases during larval development. The progressive increase of the M.L.D. also points to the same conclusion.

It is interesting to compare this finding with that of Hammett and Nowrey (4), who determined the M.L.D. by injection of As^{III} $(As_2O_3)^{III}$ for the albino rat[3] in three distinct stages of its development. They showed that susceptibility increases during the development of this animal and concluded that "these differences in susceptibility with age are attributable in large part to differences in metabolic rate",

[3] It is a curious coincidence that the M. L. D. of As_2O_3 by injection is identical for the 5th instar silkworm and the mature albino rat; *i.e.*, 8 mg. per kilo.
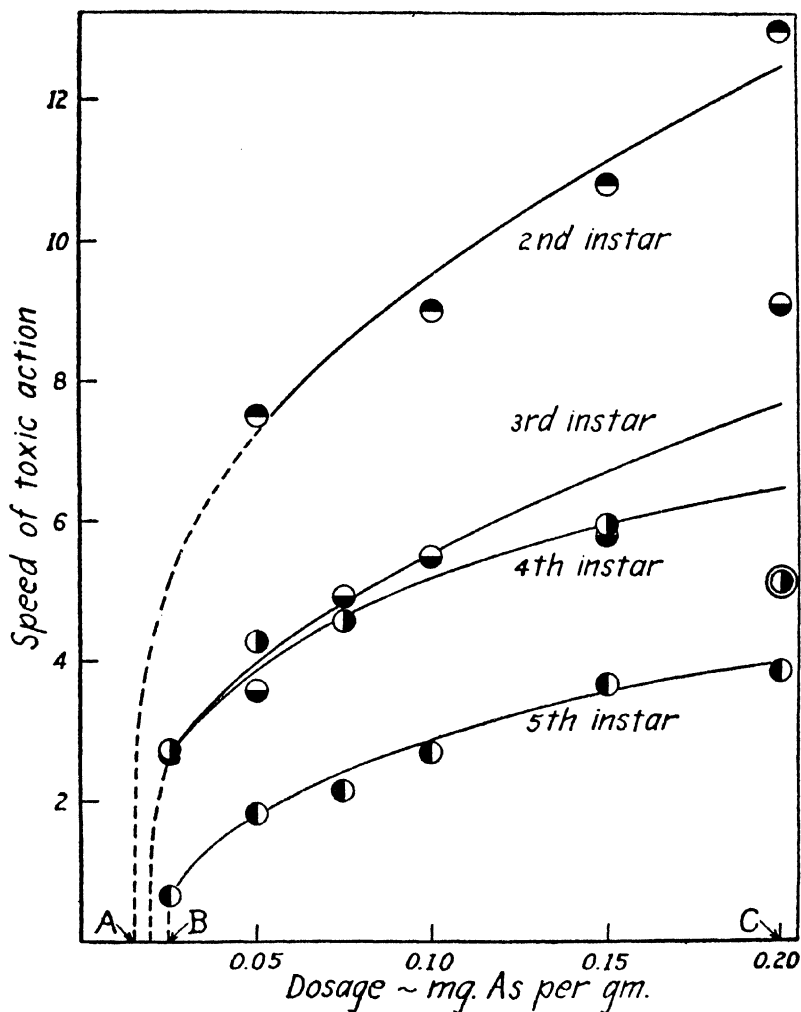
FIG. 1. The relative susceptibility of the last 4 instars of the silkworm to As[v] by mouth. Speed of toxic action (1000 ÷ survival time in min.) is plotted against dosage (mg. As per gm. silkworm). Each point represents the mean of 10 determinations of survival time. The M. L. D. increases from about 0.015 to about 0.025 mg. As per gm. during larval development. The probable values of the M. L. D. are shown by the points of intersection of the dotted lines with the X axis, but this procedure is, of course, not strictly correct, because the survival time at the M. L. D. is far from infinite.

or in other words that susceptibility varies with "the mass of active protoplasm relative to [that of] the organism as a whole". This is an attractive hypothesis, and it might easily be supposed that the relative mass of active protoplasm reaches a maximum in the mature silkworm. But Jucci (6) has shown that the velocity of growth in
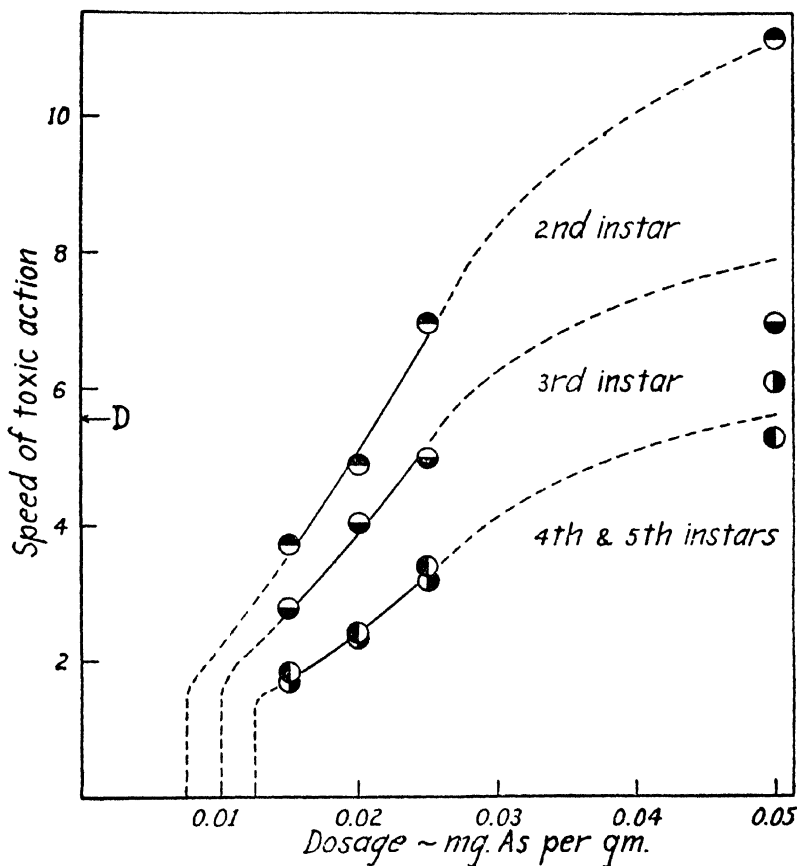
FIG. 2. The relative susceptibility of the last 4 instars of the silkworm to As[III] by mouth. Plotted like Fig. 1. Each point represents the mean of from 5 to 9 determinations of survival time. The M. L. D. increases from about 0.0075 to about 0.0125 mg. As per gm. during larval development. The curves are given a sigmoid shape, because in log plots (2) the first three points of each curve lie along parallel straight lines of slope 1.22. By comparing these curves with those of Fig. 1, the usual higher toxicity of As[III] may be observed (1).

the silkworm progressively decreases during larval development, and, if rate of growth is an index of the relative mass of metabolically active protoplasm, the explanation of Hammett and Nowrey cannot hold for the silkworm. Moreover, experiments by the writer have shown that the adult grasshopper[4] *Melanoplus femur-rubrum* is very much less susceptible to arsenic, administered either by mouth or by injection, than is the silkworm. It is probable that the metabolic activity of an adult insect is less than that of a rapidly growing larva. Fink (3) found that the rate of carbon dioxide excretion per unit body weight by larvæ of the strawberry leaf roller, *Ancylis comptana*, was nearly twice that of adults of the same species.

The argument of Hammett and Nowrey may, therefore, very well be reversed, for it is just as reasonable to suppose that speed of toxic action, and hence susceptibility, varies directly with metabolic activity. It seems probable that changes more specific than that of general metabolic rate are involved in variations of susceptibility. The gap between the 4th and 5th instar curves of Fig. 1 and the coincidence of the same curves in Fig. 2 appear to express an underlying difference in the effects of As^V and As^{III}, which does not suggest a simple, general explanation.

The form and position of the 3rd and 4th instar curves (Fig. 1) are uncertain, though the probable error of each mean survival time is less than 5 per cent of its mean. An attempt was made to check the point marked by a double circle, 2 days after the first observations were made. The first mean survival time was 196 ± 5.3 min.; the second, 132 ± 3.0 min. The difference is 64 ± 6.1 min., which is decidedly significant. It must be concluded, therefore, that the mean susceptibility of the entire population of 4th instar larvæ had changed, and that it might have changed during the 2 days necessary to determine the curves. Since it was possible that not enough attention had been paid to securing uniform cultural conditions, efforts were redoubled to maintain a plentiful fresh food supply at a fairly constant temperature. That this precaution was necessary and successful was shown by subsequent experiments to determine the frequency distribution of the survival times of 143 4th instar silkworms (2). The experiments extended over a period of 5 days and consisted of eight separate series of observations. The mean of the eight means was 264 ± 2.4 min., and the difference between the maximum and minimum means was 26 ± 8.5 min. The first mean may be said, therefore, to have been checked seven successive times.

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[4] All of six species of grasshoppers which the writer tested were found to imbibe neutral arsenical solutions avidly and without subsequent regurgitation.

IV.

Relative susceptibility, like relative toxicity, may be expressed numerically either as a ratio of the doses required to produce the same toxic effect (dosage comparison), or as a ratio of the effects produced by equal doses (effect comparison). If dosage-effect curves, like Figs. 1 and 2, are available, it is possible to express more comprehensively the relative susceptibility or relative toxicity by an "effect comparison" as the ratio of the areas under any two curves. To take a specific case (Fig. 1): the area under the 2nd instar curve, between limits A and C, divided by the area under the 5th instar curve, between limits B and C, equals 3.5. It may therefore be said that, over the dosage range studied, 2nd instar silkworms are 3.5 times as susceptible to As^V as 5th instar larvæ. The ratio for the same curves in Fig. 2 is 1.0:2.1. A "dosage comparison" could be made in the same manner by comparing areas between two curves and the Y axis and between the X axis and an arbitrary limit on the Y axis (*e.g. D*, Fig. 2); but the present data are not complete enough at lower dosages for the application of this method.

SUMMARY.

The susceptibility of the silkworm to arsenic decreases during its larval development.

Relative susceptibility may be expressed numerically as a ratio of areas under dosage-effect curves.

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THE TOXIC ACTION OF COPPER ON NITELLA.

BY S. F. COOK.

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I.

A previous investigation (Cook, 1925–26) has brought to light certain facts regarding the effect of copper on *Aspergillus niger*. In the course of that investigation, where the criterion used was the respiration of the organism, a few subsidiary experiments were performed on the respiration of *Nitella*. This plant behaved in a manner very similar to *Aspergillus*. In connection with findings obtained with the respiration method, where the product of a chemical reaction is measured, the question arises whether there is a selective action exercised by the toxic agent on the individual cells. Expressing the situation differently, do the variations in resistance among the cells affect the form of the curve of the toxic effect? There has been considerable controversy among investigators concerning this matter; the reader is referred to the discussion of the question by Brooks (1918). For this particular case it seemed desirable to secure data concerning the effect of copper, using some organism and method which would bring out clearly variations in individual resistance. For this purpose recourse was had to measurements of turgidity with *Nitella*.

The *Nitella* used in these experiments was an undetermined species found growing in a brook near Sharon, Massachusetts. (To determine the species it is necessary to obtain the plant in a fruiting condition.) The cells averaged from 1 to 3 inches in length and were uniformly about 1 mm. in diameter. They could be handled easily without injury. The material was kept in tanks of running tap water, and no evidence of deterioration was observed during several months.

In preparation for an experiment three lots of fifty cells each were placed in about 200 cc. of water until needed. Three glass jars were placed on a wire frame in a 10 gallon glass tank filled with water, in such a way that the level of the water in the tank was about 1 inch above the level of the fluid in the jars. By means of tubing run from a hot and a cold water reservoir, and a stirring device,

the temperature of the water in the tank could be varied at will or be kept constant. Thermometers were placed in the jars and in the tank and the temperature could be controlled to within about 0.1°. The reagent, copper chloride, was made up to the desired concentration and placed in the small jars. After the proper temperature had been secured the three lots of fifty cells were placed in the toxic solutions. At regular intervals the cells were lifted out, one by one, and tested. The criterion of injury was the ability of a cell to support its own weight when held by one end. This is a purely arbitrary criterion because the process by which a cell becomes less and less turgid is continuous and not sudden. The stage where a cell will no longer bear its own weight, but will collapse, is only one point on a curve which, if it could be plotted, would represent the entire process. But it is a very distinct and convenient point and may be used with the understanding that it has significance merely as an index of a definite extent of injury.

Experience with many thousand cells has brought to light no correspondence between the size and shape of a cell and readiness with which it loses its rigidity.

In order to analyze the toxic effect of a substance on an organism it is desirable to secure data on three essential aspects of the problem: (1) the course of the toxic action with time, (2) the variation of the toxic effect with the concentration of the reagent, and (3) the variation with change of temperature. These three phases will be considered separately.

II.

There are three methods of plotting a curve of toxic action. The first is the so called survivor curve, where the number or the per cent of cells surviving is plotted against time. The second is the death curve, in which the number or the per cent of cells which have died (*i.e.* lost their turgidity) is plotted against time. The third is the mortality curve where the rate of dying is the ordinate. In Fig. 1 one set of data is plotted in these three ways. If y represents the per cent surviving, the curve of the second type will be the reverse of that of the first type ($100 - y$ serving as ordinate instead of y), while the ordinate of the third type will be the first derivative of the equation of type 1 or 2. Any of these curves may be used according to circumstances.

A typical survivor curve is shown in Fig. 1, Curve A. It is evident by inspection that this is a curve of the sigmoid class; that is, of the sort expected from the assumption that the individual cells vary at random in their resistance to the action of the lethal agent. Whether

this is true remains in the realm of conjecture unless, as in cases of hemolysis or similar phenomena, the cells may be observed individually. In the present instance there is no doubt that there is a wide variation in resistance. For example, in the experiment shown in Fig. 1, Curve A the first cell collapsed let us say at 10 minutes while the last remained turgid for 4 hours. The curve then does not represent the course of a reaction but is a statistical expression. It may be used as such, and two or more such statistical curves may be compared. For although each single curve expresses the individual
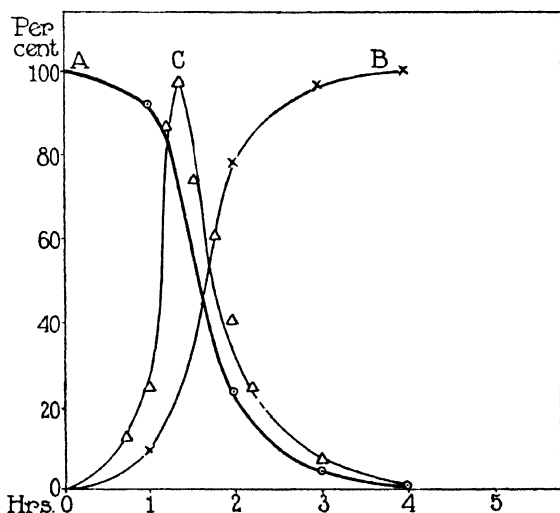
FIG. 1. Effect of copper chloride, 0.001 M, on *Nitella*. In Curve A the ordinate is the per cent surviving, in Curve B it is the per cent dead, and in Curve C it is the rate of mortality.

cell variation two such curves furnish an index of the relative power of the toxic action.

In order to analyze a curve or compare it with others we should know its equation. In the present case it is not feasible to try to deduce the equation. Consequently it is necessary to find some arbitrary and empirical equation which will fit the experimental data.

Many curves of the sigmoid type have been encountered in biological work, as for example those discussed by Brooks (1918), Fulmer and Buchanan (1923), Lotka (1923), Reed and Holland (1919), Ross

(1911), Robertson (1907, 1908), etc.   Equations of various sorts have been applied to these almost all of them involving an exponential function.   Although the fit is not absolute, the survivor curves obtained with *Nitella* are best fitted by an equation in this form:

$$y = ae^{-pt^n},$$

where y represents per cent surviving, t the time, a is a proportionality constant, and p and n are arbitrary constants.   Fig. 2 shows several
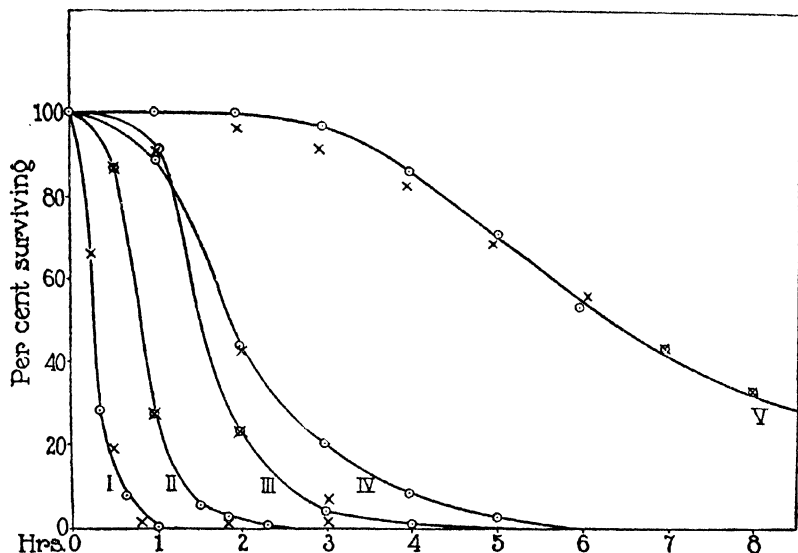
FIG. 2. Curves showing the effect of various concentrations of copper chloride on *Nitella*. The concentrations are as follows:

Curve   I 0.1 M
   "   II 0.01 M
   "   III 0.001 M
   "   IV 0.0001 M
   "   V 0.00001 M

For the equations of these curves see Table I. The roman numerals of the curves correspond to those in the table.

curves obtained with different concentrations of copper chloride and points representing equations of the above type.   It will be observed

that although the correspondence is not absolute, it is sufficiently close to justify the use of the equation for the comparison of the curves.

The time curve of the effect of copper chloride on *Nitella*, then, is of the sigmoid survivor type and can be represented by an empirical equation with a fair degree of accuracy.
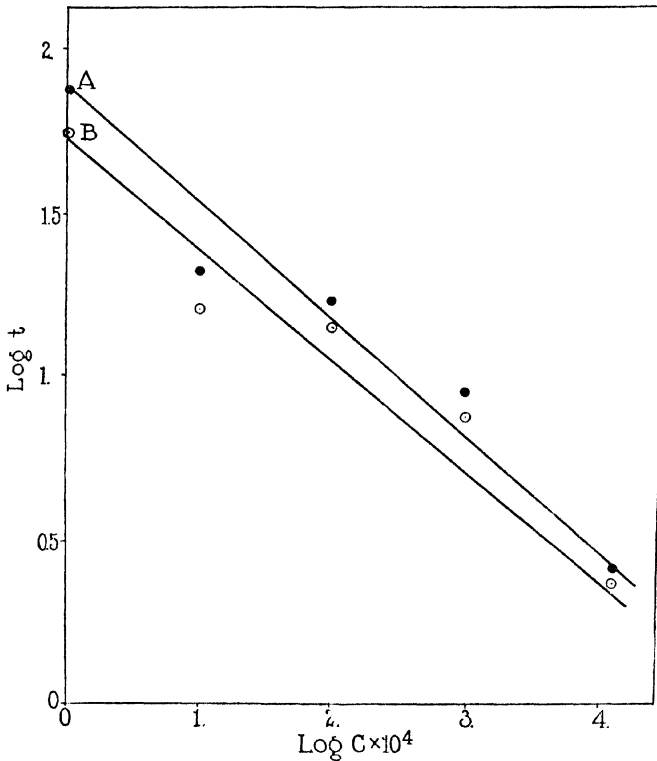
FIG. 3. Relation between concentration and time to reach a given point on the survivor curves. Curve *A* represents time to reach 40 per cent surviving and Curve *B*, 60 per cent.

III.

Fig. 2 shows the curves obtained with five concentrations of copper chloride from 0.00001 M to 0.1 M, at constant temperature. There is considerable change in toxic effect with change of concentration. In order to determine the quantitative relations several methods may be used.

1. The simplest procedure is to select some point on the ordinate and plot the logarithm of the length of time necessary to reach this point against the logarithm of the concentration. In Fig. 3 the points chosen are 60 per cent and 40 per cent. Although the points on the concentration curve are irregular, nevertheless they fall approximately on a straight line. There is, therefore, a relation between concentration and toxicity which may be expressed as:

$$\log t = - n \log C + \log a, \quad \text{or,} \quad t = aC^{-n}.$$

This relation is one commonly encountered in studies of toxicity and has been discussed in a previous paper (Cook, 1925–26).
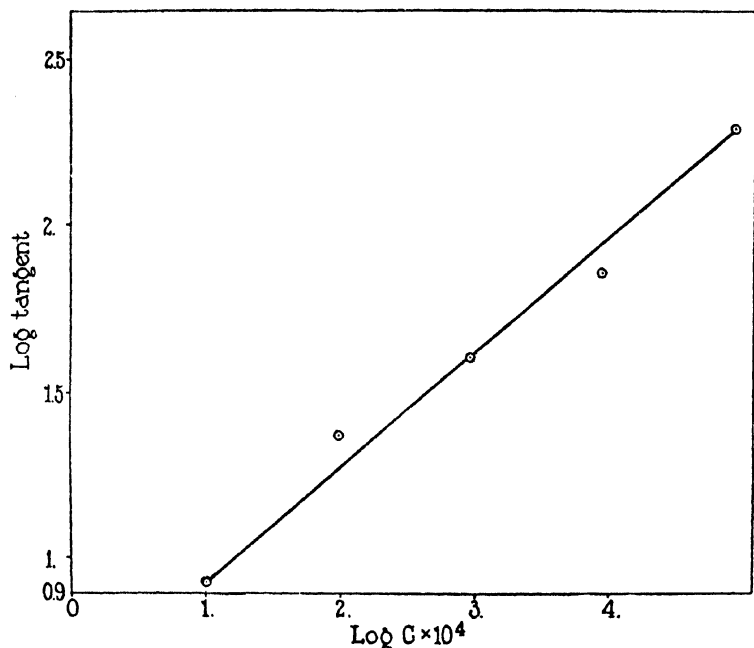
FIG. 4. Relation between concentration and the maximum tangent to the death curves.

2. Instead of considering the length of time necessary for a given percentage of the cells to collapse, it is possible to consider the rate of mortality at the moment of most rapid killing. The slope of the survivor curve being an expression of the rate, we may find the tangent to the curve at the steepest point. For this purpose it is better to use a curve of the type of Fig. 1, Curve *B*—the death curve—the reason being that while the value of the tangent will be the same in either type *A* or *B*, in type *B* it is positive whereas in type *A* it is negative. If this value

of the tangent to the steepest point of the experimental curves is obtained by means of suitable instruments, and the logarithm of the tangent is plotted against the logarithm of the concentration, Fig. 4 is the result. Like the preceding curve, it is linear and indicates the following relation:

$$\log \tan y = n \log C + \log a, \quad \text{or} \quad \tan y = aC^n,$$

where $\tan y$ is the tangent to the curve at its steepest point, or in other words is the maximum tangent to the curve. But $\tan y$ is equivalent to $\dfrac{dy}{dt}$ (at its maximum) in an equation relating y to t, and simply states that the maximum rate of toxic action is proportional to some power of the concentration.

3. Instead of the actual experimental curves the equations of the curves may be used, and here again the concentration may be related to the time in which a given amount of activity occurs or to the maximum rate of the action. The most convenient point to select for the time value is that time necessary for the maximum rate to be reached, or the point on the curve at which the tangent has its maximum value. (It will be noted in passing that this point of greatest activity falls in all the curves at approximately the same distance along the ordinate from the intersection with the abscissa. Using the values of y, it falls at about 50 per cent, or at the time when half the cells have been killed.)

Consider the equation $y = ae^{-pt^n}$. This is based on the curve of type A, the survivor curve. In order to keep the sign positive it is better to use type B, where

$$y = 100 - ae^{-pt^n}. \tag{1}$$

Then $\dfrac{dy}{dt} = apnt^{(n-1)} e^{-pt^n}. \tag{2}$

When the rate is at a maximum $\dfrac{dy}{dt}$ will be at a maximum and $\dfrac{d^2y}{dt^2}$ will equal zero.

But $\dfrac{d^2y}{dt^2} = ap \left[(n-1)t^{n-2} e^{-pt^n}\right] - apnt^{n-1} (-pnt^{n-1} e^{-pt^n}).$

Simplifying, we have $\dfrac{d^2y}{dt^2} = npt^{2(n-1)} - (n-1)t^{n-2}. \tag{3}$

When $\dfrac{dy}{dt}$ is at a maximum and $\dfrac{d^2y}{dt^2} = 0$, then

$$npt^{2(n-1)} = (n-1)t^{n-2}.$$

Solving, $t = \sqrt[n]{\dfrac{n-1}{np}}. \tag{4}$

For any equation the constants n and p are determined and we may solve for t. This gives the length of time necessary for the tangent to attain its greatest value, or for the lethal action to be at its maximum. To obtain an expression for the amount of action this value of t may be substituted in (2) and the result will be the value of $\dfrac{dy}{dt}$ which is the equivalent of tan y (at its maximum) in the experimental curves. Table I summarizes these values.

Fig. 5 shows the logarithm of the concentration plotted against the logarithm of t and $\dfrac{dy}{dt}$. In both instances the curves are linear. Therefore

$$\log t = -n \log C + \log a \quad \text{or}$$

$$t = aC^{-n} \quad \text{and}$$

$$\log \frac{dy}{dt} (\text{max.}) = n \log C + \log a \quad \text{or}$$

$$\frac{dy}{dt} (\text{max.}) = aC^{n}.$$

TABLE I.

Concentration.	y	$\dfrac{d^2y}{dt^2}$	$\dfrac{dy}{dt}$ (max.)
0.1 M	$100\,(1-e^{-7t^{2.1}})$	0.303	223.8
0.01 M	$100\,(1-e^{-1.2t^{3}})$	0.7051	117
0.001 M	$100\,(1-e^{-0.09t^{4}})$	1.699	83.3
0.0001 M	$100\,(1-e^{-0.09t^{3.2}})$	1.889	58.3
0.00001 M	$100\,(1-e^{-0.008t^{2.4}})$	5.655	30.2

The exponent n or $-n$ has nearly the same value in all these curves.

In Fig. 3. Curve A $-n = 0.336$
" 3.   " B $-n = 0.332$
" 4. $n = 0.340$
" 5.   " A $-n = 0.325$
" 5.   " B $n = 0.222$

The uniformity with which the value $0.325 - 0.340$ occurs is significant evidence that we are dealing with one and the same process in the cell whether we consider it from the point of view of time or of intensity of toxic action, by means of the experimental curves or by means of empirical equations.

Any one of these methods of comparison will lead to the same result. The conclusion is therefore justified that the toxic effect of the copper chloride varies as a constant, fractional, power of its concentration.

This relation is of importance because it is analogous to that which holds in the case of respiration (*Aspergillus niger;* Cook, 1925–26) and demonstrates that although the time curves obtained with the two criteria are very different, yet in some fundamental way the toxic
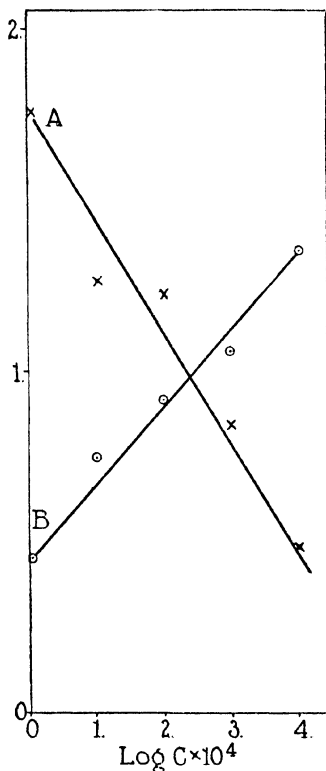
FIG. 5. Relation between concentration and the equations representing the death curves. In Curve *A*, log *C* is plotted against the logarithm of $\frac{dy}{dt}$ at its maximum and in Curve B, log *C* is plotted against the logarithm of the time for $\frac{dy}{dt}$ to reach its maximum.

action of the metal is the same with both organisms. Just how fundamental is the similarity is evidenced by the fact that the power relation is found very frequently in other work on toxicity.

IV.

When the temperature instead of the concentration is varied, we find that with increase in temperature the time necessary to kill a constant percentage of the cells becomes shorter. In Fig. 6 the logarithm of the reciprocal of this time is plotted against the reciprocal of the absolute temperature. The result is a series of intersecting curves. The reciprocal of the time necessary to reach a definite point
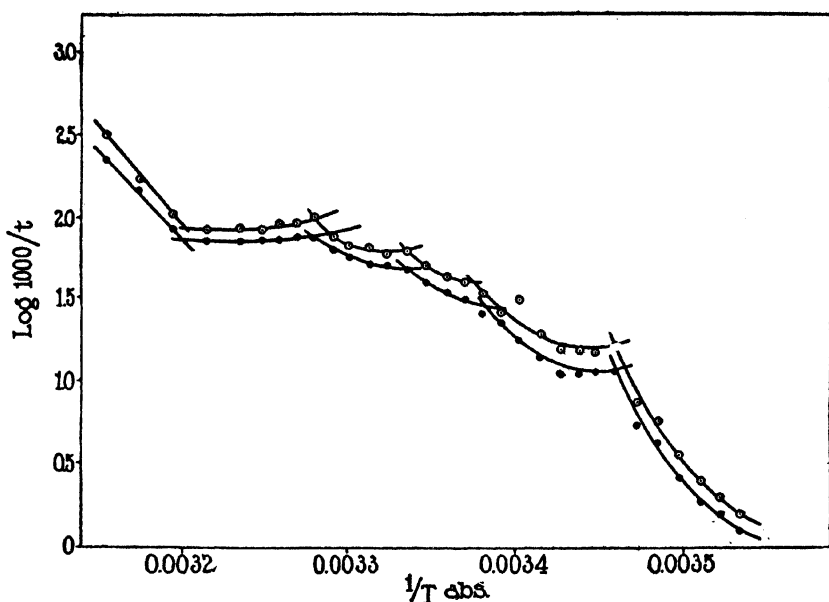
FIG. 6. The effect of varying the temperature. The reciprocal of the absolute temperature is plotted against log $\dfrac{1000}{t}$ where, in the upper curve, t is the time necessary to reach 60 per cent surviving and, in the lower curve, 40 per cent.

in the toxic action is equivalent to the activity of the toxic agent at a given temperature.

It will be observed that the slope of the curves falls off with rising temperature till about 32°, above which there is little change in toxicity with temperature (if any it is negative) up to 39°. Then the points rise rapidly, and in a linear fashion. This rise doubtless is due to the physical effects of the heat, such as coagulation of proteins etc. Evi-

dence in favor of this supposition is the high value of μ (about 40,000). In the lower range of temperatures the points of intersection (or cusps) fall quite definitely at 16°, 27°, and 32°. In the vicinity of 23–24° there is another break, but owing to the irregularity of the data it appears to fall at 23° in one case and at 24° in the other. In general breaks are known to occur quite frequently at these temperatures. In a recent paper Crozier (1925–26) has assembled data which show that the points where breaks occur most often include 15°, 27°, and 30°. Cases are also known where a break occurs at 23°.

The formula for the effect of temperature on a chemical reaction as found by Arrhenius is:

$$\frac{K_2}{K_1} = e^{\frac{\mu}{2}\left(\frac{1}{T_1} - \frac{1}{T_2}\right)},$$

where K_1 and K_2 are velocity constants, T_1 and T_2 the corresponding absolute temperatures, and μ is a constant which is characteristic of the system and ideally represents the heat of activation. When the logarithm of K is plotted against $1/T$ abs. (and here log $1/t$ is equivalent to log K) one or more straight lines are usually obtained. Crozier (1924–25) has recently plotted the data for a great many biological activities and has found that there are often two or more such lines which intersect at rather definite points on the temperature scale (e.g., 15–16°). For a fuller discussion of the situation, reference should be made to his papers. Usually straight lines are obtained. Lillie,[1] however, has obtained data with sea urchin eggs in which the points for parthenogenesis with acid fall along curved lines similar to those here obtained with copper.

The question has been raised whether the so called straight lines are in fact geometrically straight or are curved so slightly that the curvature cannot be detected by ordinary methods. If this is true, and all temperature plots are really curves, then between the usual cases and the one here recorded there is a difference not in principle but only in degree. But for present purposes it is not necessary to decide this question. Since the curvature of the ordinary lines is

[1] Quoted by Crozier (1924–25), p. 193. Crozier has plotted the data published by Lillie.

so slight as to defy detection they may be considered straight, and the effect of copper on *Nitella* may be held an exception to the general rule.   On this basis the action of copper must involve some process or state which is not present in the normal vital processes.   It is worth while to try to gain some conception of the situation underlying these temperature effects by setting up a hypothetical normal system and by assuming an appropriate mode of action of the copper.   There would be no proof that such a hypothetical system actually exists. It would simply be shown that if such a system were present the effect of temperature variation would be such as that observed experimentally.

Let us begin by assuming that any vital activity (A) which may be under consideration depends on a reaction or a series of reactions having a velocity M_1.   There is also a reaction in the reverse sense with a velocity M_2.   The exact nature of these reactions is immaterial. The essential point is that the intensity or rate of the activity, A, depends on the net velocity of the forward reaction.   The reverse reaction nullifies the effect of the forward reaction and therefore the net intensity of A which is observed bears a definite ratio to the net velocity of the forward reaction.   The net velocity is the velocity M_1 minus the velocity of the reverse reaction, M_2.   If M_1 is equal to, or less than, M_2 there will be no observed activity.   Hence M_1 must always be the larger.

Now suppose that the value of M_1 depends on a catalyst B while that of M_2 depends on another catalyst Y.   Then if B and Y vary, M_1 and M_2 will vary, and A will vary correspondingly.   Since this system represents a very general case and is not intended to fit any particular instance it is made as simple as possible, but with the understanding that it might be extended considerably as occasion might require.

In varying B and Y it is assumed that each is the intermediate product of a series of consecutive reactions, thus:

$$P \xrightarrow{K_1} B \xrightarrow{K_2} C \quad \text{and} \quad P \xrightarrow{K_3} Y \xrightarrow{K_4} Z.$$
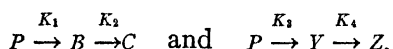
If P is very great in quantity with respect to B, C, Y, and Z, then the quantity of B and Y will depend on the values of the velocity con-

stants K_1, K_2, K_3, and K_4. Thus if K_1 is increased while K_2 remains constant, B will be formed more rapidly until the concentrations come to equilibrium in accordance with the equation $PK_1 = BK_2$. The same holds true for $PK_3 = YK_4$. Similarly, if K_2 or K_4 is increased B and Y will decrease.

What, now, will be the effect of varying the temperature? In each step of these reactions the velocity constant will be determined by the critical thermal increment (μ) of that step. For instance let us assume that at $0°C$. K_1, K_2, K_3, and K_4 each equals unity. Then if P is indefinitely large it will remain practically constant throughout the temperature range here considered ($0°$ to $50°$). It may therefore be disregarded numerically and the equations may be reduced to:

$$B = \frac{K_1}{K_2} \quad \text{and} \quad Y = \frac{K_3}{K_4}.$$

Then, since at $0°$ all the constants equal unity, $B = Y = 1$, $B - Y = 0$, and $M_1 - M_2 = 0$. This indicates that the observed vital activity, A, ceases at $0°$. Certainly most activities cease at that temperature, and if they continue the rate must be exceedingly slow.

As the temperature rises the ratios $\frac{K_1}{K_2}$ and $\frac{K_3}{K_4}$ will no longer equal unity since the critical thermal increments are not the same for each. If, in any one instance, we take 10 degree intervals then the formula of Arrhenius may be written:

$$\frac{K}{K_1} = e^{\frac{\mu}{2}\left(\frac{10}{T_1 T_2}\right)}$$

The left-hand term, $\frac{K_2}{K_1}$, is often expressed as Q_{10}, and represents the increase in velocity for a rise of 10 degrees. Q_{10} will itself vary depending on the absolute temperature, and if at $0°$ the velocity constants equal unity, their values for other temperatures will be as shown in Table II.

Now assume the following two sets of values for the critical thermal increments:

I.	II.
For $K_1, \mu = 6,300$	For $K_1, \mu = 14,000$
$K_2, \mu = 1,540$	$K_2, \mu = 6,300$
$K_3, \mu = 14,000$	$K_3, \mu = 22,000$
$K_4, \mu = 1,540$	$K_4, \mu = 6,300$

By means of the equations $B = \dfrac{K_1}{K_2}$ and $Y = \dfrac{K_3}{K_4}$ the values of B and Y may now be calculated for 10 degree intervals starting with $0°$.

TABLE II.

Values of Q_{10} for different critical thermal increments.

Temperature.	$\mu = 1,540$	$\mu = 6,300$	$\mu = 14,000$	$\mu = 22,000$	$\mu = 28,000$	$\mu = 34,000$
°C.						
0–10	1.104	1.503	2.47	4.16	6.12	8.91
10–20	1.097	1.462	2.32	3.76	5.41	7.76
20–30	1.091	1.424	2.20	3.45	4.86	6.78
30–40	1.085	1.397	2.10	3.22	4.43	6.11
40–50	1.078	1.350	1.98	2.96	3.95	5.31

Values of K for different critical thermal increments.

Temperature.	$\mu = 1,540$	$\mu = 6,300$	$\mu = 14,000$	$\mu = 22,000$	$\mu = 28,000$	$\mu = 34,000$
°C.						
0	1.000	1.000	1.000	1.000	1.000	1.000
10	1.104	1.503	2.47	4.16	6.12	8.91
20	1.211	2.197	5.73	15.64	33.11	69.14
30	1.313	3.128	12.6	53.96	160.9	468.7
40	1.422	4.38	26.5	173.7	712	2863
50	1.53	5.91	52.5	514.2	2812	15174

They are shown in Table III. (The values for $15°$, $25°$, and $35°$, printed in italics, are obtained by plotting the curves of $\dfrac{K_1}{K_2}$ etc. and interpolating. They are of course less accurate than the calculated values.)

By subtracting Y from B the values of the net velocity M_1, or the rate of the observed activity, may be obtained. These values are

also given in Table III. If the logarithm of $(B - Y)$ is plotted against the reciprocal of the absolute temperature Fig. 7, Curves I and II, is the result. It will be seen that there are two intersecting lines. It cannot be maintained that the lines are geometrically straight, but they are as straight as those obtained from experimental data which is as far as it is necessary to carry the analogy. Since $B - Y = 0$ at $0°$ it is obvious that at the extreme right the line must be curved, but it is also evident that if any observed vital process ceases at $0°$ the line representing it, also, must curve as it approaches $0°$. Only the range from $10°$ to $50°$ need be considered here.

TABLE III.

Temperature.	I.			II.		
	B	Y	$B - Y$	B	Y	$B - Y$
°C.						
0	1.000	1.000	0.000	1.000	1.000	0.000
10	2.245	1.363	0.882	2.773	1.646	1.127
15	3.1	1.55	1.55	4.5	2.05	2.45
20	4.735	1.733	3.002	7.109	2.604	4.505
25	6.7	2.0	4.7	11.0	3.25	7.75
30	9.618	2.388	7.230	17.23	4.02	13.21
35	13.3	2.7	10.6	26	5	21
40	18.66	3.084	15.58	39.65	6.05	33.6
50	34.31	3.863	30.45	87.1	9.5	76.6

A system has thus been set up which may be diagramed as follows:
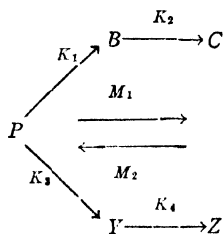
We have next to enquire how, by the introduction of a toxic agent, the straight lines may be converted into curves. For the sake of convenience we will use copper, although many other substances might have the same effect. The suggestion has been made in a previous

paper (Cook, 1925–26) that copper is activated in the cell by entering into a reversible combination with a cell constituent such as T. This reaction has the form $Cu + T \rightleftarrows CuT$. In considering the effect of temperature we may assume that at constant temperature the concentration of T is constant at, say, 1, while that of the copper is so
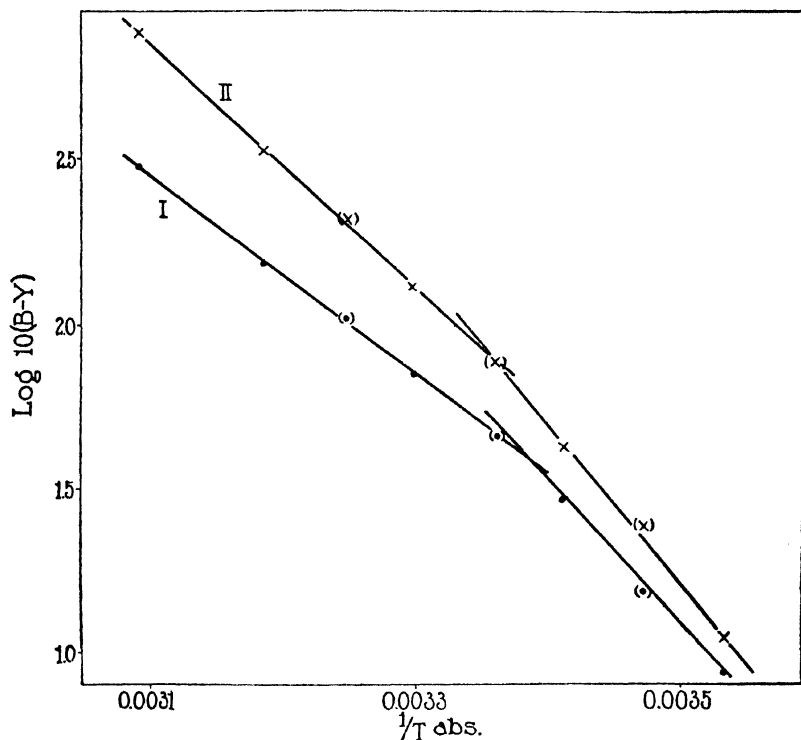
Fig. 7. Theoretical temperature curves using the data for $(B - Y)$ as given in Table III. The roman numerals correspond with those in the table. The bracketed points are those obtained by interpolation in the calculated curves for $\dfrac{K_1}{K_2}$, etc.

much greater that it remains practically unchanged when T varies. The reaction may then be treated as an equilibrium between T and CuT. If there is another substance, V, with which the copper combines, and which is present in the same concentration as T, there will

be two forms of activated copper, CuT and CuV. The amount present of each will depend on the velocity constants of the respective forward and reverse reactions:

$$Cu + T \underset{K_2}{\overset{K_1}{\rightleftarrows}} CuT \quad \text{and} \quad Cu + V \underset{K_8}{\overset{K_7}{\rightleftarrows}} CuV$$
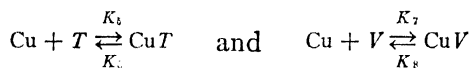
But T and V are limited. The amount of each, whether by itself or in combination, cannot, let us say, exceed 1.

TABLE IV.

Temperature.	1	2	3	4	5	6	7
°C.							
0	1.000	1.000	0.500	0.001	0.250	0.250	0.200
5	1.18	1.15	0.445	0.003	0.252	0.28	0.208
10	1.471	1.363	0.400	0.008	0.314	0.354	0.264
15	1.75	1.55	0.36	0.017	0.372	0.442	0.332
20	2.117	1.733	0.321	0.054	0.517	0.607	0.485
25	2.51	2.00	0.286	0.130	0.568	0.685	0.540
30	2.980	2.388	0.251	0.261	0.587	0.712	0.562
40	4.092	3.084	0.196	0.669	0.772	0.924	0.752
50	5.47	3.86	0.154	0.908	1.23	1.40	1.21

Column 1: values of B when $K_1 = 1$ at $0°$ with $\mu = 28,000$ and $K_2 = 1$ at $0°$ with $\mu = 22,000$.

Column 2 : values of Y when $K_3 = 1$ at $0°$ with $\mu = 6,300$ and $K_4 = 1$ at $0°$ with $\mu = 1,540$.

Column 3 : values of CuT when $K_5 = 1$ at $0°$ with $\mu = 22,000$ and $K_6 = 1$ at $0°$ with $\mu = 28,000$.

Column 4 : values of CuV when $K_7 = 0.001$ at $0°$ with $\mu = 34,000$ and $K_8 = 1$ at $0°$ with $\mu = 1,540$.

Column 5 : values of $B - Y + 0.5\,CuT - 0.5\,CuV$.

Column 6 : values of $B - Y + 0.5\,CuT - 0.5\,CuV$ when K_5 and K_6 are equal and have the same thermal increment.

Column 7 : values of $B - Y + 0.4\,CuT - 0.5\,CuV$.

Then if $\qquad T = \dfrac{CuTK_6}{K_5} \quad$ and $\quad CuT + T = 1,$

$$1 - CuT = \frac{CuTK_6}{K_5},$$

Rearranging, $\mathrm{Cu}T = \dfrac{K_5}{K_5 + K_6}$,

and, similarly, $\mathrm{Cu}V = \dfrac{K_7}{K_7 + K_8}$.

If to K_5, K_6, K_7, and K_8 are assigned appropriate values at $0°$, and each has its corresponding critical thermal increment, we can calculate the amount of $\mathrm{Cu}T$ and of $\mathrm{Cu}V$ present at any temperature. Table IV gives some of these values.
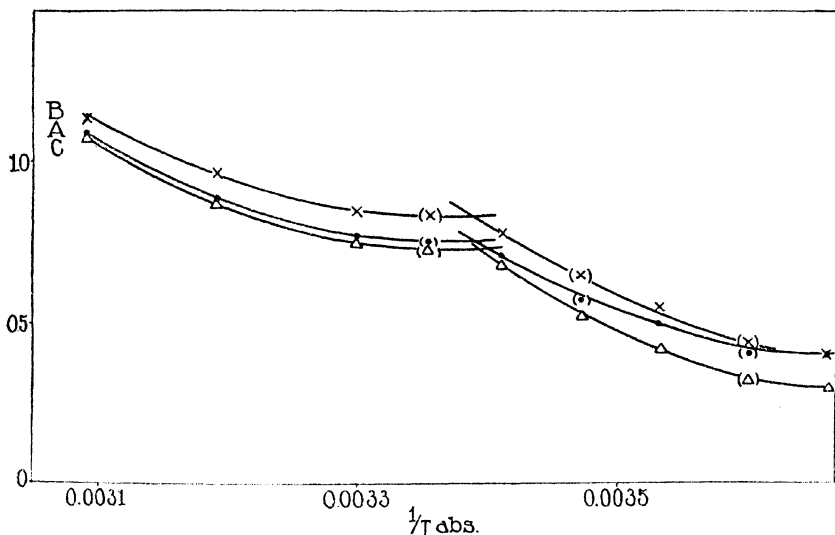
FIG. 8. Theoretical temperature curves using the data in Table IV. Curve A is based on Column 5 in Table IV and the ordinate is the logarithm of $B - Y + 0.5\ \mathrm{Cu}T - 0.5\ \mathrm{Cu}V$. Curve B is based in a similar way on Column 6 and Curve C on Column 7.

The mode of action of the activated copper is assumed to be the following: The activated copper acts as a catalyst, not altering or inhibiting the normal reactions which form B and Y, but acting as a secondary catalyst to further catalyze the reactions designated by M_1 and M_2. $\mathrm{Cu}T$ acts in conjunction with B to alter the value of M_1 and $\mathrm{Cu}V$ acts with Y to affect M_2. Then in general the expression for the ordinate is: $\mathrm{Cu}T + B - Y - \mathrm{Cu}V$. It is not necessary that $\mathrm{Cu}T$ and $\mathrm{Cu}V$ should have the same catalytic power as B and Y. It

is possible for them to be more or less powerful when present in the same quantity. Thus CuT might be one-half as effective as B. Then instead of adding CuT and B we would add $0.5\ CuT$ and B. Similarly CuV might be one-half as powerful a catalyst as Y and we would subtract $0.5\ CuV$. Using these figures the above expression becomes:

$$0.5\ CuT + B - Y - 0.5\ CuV.$$

Using these figures we obtain Fig. 8, Curve A. Table IV gives data for three such curves (plotted in Fig. 8). It will be observed that these lines are unquestionably curved, and resemble in principle the results obtained with copper chloride and *Nitella*. No attempt is made to precisely duplicate the experimental case.

The system outlined above is rather complex, but there is no reason to believe that what actually occurs is any less so. In fact it is likely to be much more complicated. A hypothetical system of this sort does not pretend to give a picture of what goes on in the cell in all its details. It merely indicates that if some such system *were present* it would give curves similar to those actually secured. At the same time it furnishes a tangible representation of the vital processes, a sort of working foundation on which we may build a more substantial edifice of fact. This is its main justification.

SUMMARY.

1. Using the loss of turgidity of the cells as a criterion it is found that the toxicity curve of copper chloride with *Nitella* is sigmoid. An empirical equation can be constructed which will approximately fit the curve.

2. When the concentration of the copper chloride is varied the toxic effect varies as a constant, fractional, power of the concentration. This relation holds when the concentration is plotted against either (1) the time necessary to reach a given point on the ordinate of the survivor curve, (2) the maximum speed of toxic action as shown by the tangent to the survivor curve or (3) the first derivative of the equation which fits the survivor curve.

3. When the temperature is varied and the logarithm of the reciprocal of the time necessary to reach a given point on the survivor

curves is plotted against the reciprocal of the absolute temperature the resulting figure consists of several intersecting curves. A hypothetical system is described which will give straight lines under normal conditions and curves when acted upon by a toxic agent.

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THE SIZE OF PORES IN COLLODION MEMBRANES.

By DAVID I. HITCHCOCK.

(From the Laboratories of The Rockefeller Institute for Medical Research.)

In a study of collodion membranes which had been coated with films of gelatin or egg albumin, it was found that the presence of the protein reduced the rate of flow of water through the membranes, as would be expected if the protein film were formed on the walls of the pores.[1] In order to determine the thickness of such films, an attempt has been made to estimate the size of pores in membranes not treated with protein.

The method which was selected as giving the most reasonable and definite results appears to be also the oldest, having been used in 1872 by Guérout.[2] It depends on the assumptions that the membrane acts like a bundle of capillary tubes having a length equal to the thickness of the membrane, and that the law of Poiseuille applies to the flow of water through such short and narrow tubes. The quantities measured are the thickness of the membrane, the rate of flow of water, under a known pressure, through a fixed area of the membrane, and the relative volume occupied by the water in the wet membrane.

Determination of Fractional Volume of Pores.

Membranes of varying permeability were prepared by the method previously described,[1] which is essentially that of Bartell and Carpenter.[3] The permeability was fixed by the time during which the organic solvents were allowed to evaporate before the addition of water. Each large membrane, after at least 1 day in water, was cut into uniform disks with a steel die 3.81 cm. in diameter. The water content of each membrane was determined by weighing several such

[1] Hitchcock, D. I., *J. Gen. Physiol.*, 1925–26, viii, 61.

[2] Guérout, A., *Compt. rend. Acad.*, 1872, lxxv, 1809.

[3] Bartell, F. E., and Carpenter, D.C., *J. Phys. Chem.*, 1923, xxvii, 252.

disks, after blotting between filter papers, in a closed weighing bottle, and then drying to constant weight at 100°C. The thickness was measured, as before, by means of a microscope provided with a micrometer eyepiece, nine readings being made with each membrane.

When the ratio of wet weight to dry weight, w, was plotted against the thickness in cm., l, the points fell close to a straight line. Such a linear relation was previously found by Walpole,[4] who deduced the equation

$$l = m \left(w - 1 + \frac{1}{d} \right).$$ (1)

Here l is the thickness of the membrane, m the number of gm. of dry collodion per sq. cm. of wet membrane and d is the density of collodion. The equation follows from the assumptions that the volume of the wet membrane is the sum of the volumes of water and collodion contained in it, and that the density of the collodion itself remains constant in all the membranes. Three determinations were made of the density of the collodion used in the present work, the samples used being a hard lump of air-dried collodion, a membrane of low permeability, and a membrane of high permeability. The values obtained were 1.65, 1.65, and 1.66. The determinations were made at 20°C. with a specific gravity bottle. If Walpole's law of additive volumes did not apply, the figures could not be identical. On testing Walpole's equation with the experimental values for d, m, and w, it was found that the calculated values for l agreed well with those observed.

On the basis of this idea of additive volumes, the fraction of the membrane volume occupied by water, f, is given by the relation

$$f = \frac{m}{l}(w - 1),$$ (2)

in which the quantities determined experimentally are the thickness and the wet and dry weights of a known area of membrane.

The assumption involved in this calculation, that all the water in the blotted membranes is in the pores and not combined with the collodion, was roughly confirmed in the following way. Measurements were made of the expansion produced on freezing wet membranes, by

[4] Walpole, G. S., *Biochem. J.*, 1915, ix, 284.

a dilatometer method similar to that described by Foote and Saxton.[5] The results indicated that 85 per cent of the water in a blotted membrane of the most permeable type studied in this work froze above $-6°C.$, and that no further freezing took place on cooling to $-18°C.$

Values for the fractional pore volume have been obtained in another way, based on measurements of electrical conductivity. The electrodes were platinized platinum disks forming the ends of a closed cylinder whose sides were glass tubes. The cell was filled with $N/50$ KCl, and the membrane, after being soaked in the same solution, was supported across the cylindrical space between the electrodes by means of the ground ends of the tubes forming the sides of the cell. The conductivity at 30°C. was measured in the usual way, the source of current being an audio oscillator of 1000 cycles. It was necessary to

TABLE I.

Fraction of Membrane Volume Occupied by Pores, As Determined by Wet and Dry Weight and by Conductivity.

Designation of membrane.......	B 6	B 1	B 8	B 4	B 2	B 7	B 5	B 11	B 3	B 10
f (by weight).............	0.92	0.97	0.89	0.85	0.77	0.62	0.69	0.63	0.41	0.54
f (by conductivity).......	0.90	0.88	0.78	0.79	0.76	0.72	0.64	0.46	0.25	0.11

balance the rather large capacity of the cell, especially when the membrane was in place, by means of variable condensers. The differences in resistance observed with and without the membrane were not large, except in the case of the least permeable membranes, and in the latter case the readings were not well reproducible with different pieces of the same membrane. Accordingly the results of the measurements are given, not as being of much value in themselves, but simply as confirmatory of the order of magnitude of the pore volumes. The latter were calculated on the assumption of cylindrical pores and of additive resistance for the layers of solution inside and outside of the membrane. The relative pore volumes obtained by the two methods are given in Table I.

[5] Foote, H. W., and Saxton, B., *J. Am. Chem. Soc.*, 1916, xxxviii, 588; 1917, xxxix, 627, 1103.

Determination of Pore Size from Poiseuille's Law.

The measurement of the rate of flow of water through the membranes has been described elsewhere.[1]   The apparatus was not in a thermostat, but the temperature of the water varied from 18.5 to 24°C. in different experiments.   Correction was made for the effect of temperature on the viscosity of water by means of Bingham's data.[6] From four to eight disks of the same membrane were used for successive readings, the average being taken.   The highest pressure used was 35 cm. of Hg.   The membranes were all visibly stretched to some extent, and the stretching was neglected in the calculation of the rate of flow per sq. cm.   The data were reduced to c. g. s. units, the permeability, Q, being defined as the number of cc. of water flowing in 1 second through 1 sq. cm. of membrane under a pressure of 1 dyne per sq. cm.   (This differs from the notation of the previous paper.[1]) On the assumption that the membrane behaves like a bundle of capillary tubes of length equal to its thickness, the rate of flow should be given by Poiseuille's law in the form

$$Q = \frac{n \pi r^4}{8 l \eta}. \tag{3}$$

In this equation the unknown quantities are n, the number of capillaries per sq.cm., and r, the pore radius in cm.   The quantities Q and l, as defined above, were determined experimentally, while values for η, the viscosity of water at the temperature of the experiment, were taken from the data of Bingham.[6]   Thus the equation was solved for values of $n \pi r^4$.   The fraction of the membrane volume occupied by pores, f, must on the assumption of cylindrical pores be equal to $n \pi r^2$, and the determination of this quantity has already been discussed.   Hence the value of r, the pore radius, should be given by

$$r = \sqrt{\frac{n \pi r^4}{n \pi r^2}} = l \sqrt{\frac{8 \eta Q}{m (w - 1)}}, \tag{4}$$

while that of n, the number of pores per sq. cm., should be

$$n = \frac{n \pi r^2}{\pi r^2} = \frac{m^2 (w - 1)^2}{8 \pi Q l^3 \eta}. \tag{5}$$

[6] Bingham, E. C., Fluidity and plasticity, New York and London, 1922.

The values so obtained are given in Table II, together with the data from which they were calculated.

On plotting the values of r against those for l, the points fell close to a straight line, which may be represented by the empirical equation

$$r = 1.2 \times 10^{-4}\, l. \tag{6}$$

TABLE II.

Calculation of Pore Radii from Measurements of Thickness, Wet and Dry Weight, and Rate of Water Flow.

Designation of membrane.	Thickness, in cm., $l, \times 10^{4}$.	Gm. collodion per cm.^{2}, $m, \times 10^{4}$.	Gm. water per gm. collodion, $w - 1$.	Rate of flow in c.g.s. units, $Q, \times 10^{9}$.	Pore radius in cm., $r, \times 10^{6}$.	No. of pores per cm.^{2}, $n, \times 10^{-10}$.
B 15	21.4	2.45	8.07	2.48	2.08	7
B 6	19.6	2.40	7.54	2.46	2.01	7
B 1	18.6	2.21	8.16	3.03	2.20	6
B 22	17.5	2.39	6.31	2.05	1.80	8
B 8	11.8	2.22	4.72	1.97	1.43	14
B 4	11.1	2.35	4.00	1.98	1.39	14
B 2	11.1	2.28	3.74	1.53	1.31	14
B 17	10.2	2.34	3.29	1.39	1.21	16
B 20	8.6	2.36	2.56	1.16	0.99	26
B 13	8.5	2.38	2.82	1.20	1.02	24
B 7	8.4	2.30	2.25	1.09	1.07	17
B 12	7.1	2.18	2.12	0.673	0.75	37
B 5	6.8	2.24	2.09	1.23	0.97	23
B 21	6.0	2.44	1.03	0.408	0.63	41
B 11	4.6	2.16	1.32	0.585	0.57	61
B 23	3.3	2.55	0.59	0.278	0.40	89
B 14	3.2	2.33	0.59	0.166	0.31	140
B 3	2.9	2.24	0.52	0.247	0.36	100
B 19	2.7	2.39	0.45	0.103	0.24	220
B 10	2.4	2.16	0.60	0.284	0.32	170
B 18	2.2	2.38	0.53	0.236	0.27	270

Likewise an approximately linear relation was found to exist between the observed values of Q and w, according to the empirical equation

$$Q = 4.3 \times 10^{-10}\, (w - 1). \tag{7}$$

It is possible to calculate the pore radii from the values of f obtained from conductivity data. The values so obtained are still of the same order of magnitude, even where the values for f differ widely. Thus,

for membrane B 10, where the divergence is greatest, the conductivity method gives $r = 0.69 \times 10^{-6}$ instead of 0.32×10^{-6}. By making use of Walpole's relation, equation (1), the radii may be calculated from the measurements of l and Q, or w and Q. By using the empirical relations given in equations (6) and (7) together with equation (1), the radii may be calculated from a single measurement, either of l, Q, or w. The results do not differ materially from those given in Table II.

The values for n given in Table II are not constant, but show a marked increase as the permeability of the membrane decreased. This is contrary to the conclusion of Bartell and Carpenter,[3] which was adopted by the writer in a previous paper.[1] It happens, however, that the variation of n is such that the relation between the observed amounts of adsorbed gelatin and the calculated relative pore surface is still linear.

Microscopic Structure of Collodion Membranes.

Fig. 1.

Samples of the membranes used in the present work were examined under the microscope with transmitted light, but showed no definite evidence of structure even at a magnification of 1000 diameters with an oil immersion objective. With dark-field illumination, however, distinct bright granules were visible with much lower magnification. Fig. 1 was made from a photograph of a fragment of collodion which was cut by hand from a wet permeable membrane and mounted in water. The magnification was about 430 diameters, obtained with a 16 mm. objective and a No. 18 Zeiss ocular, the illumination being that from a Zeiss dark-field condenser. All the membranes so examined had a similar appearance; it made no apparent difference whether the membrane was permeable or impermeable, whether the sections were cut with a microtome from membranes imbedded in paraffin, whether they were cut by hand from wet membranes, or whether the microscope was simply focussed on the surface of an ordinary thick membrane. Staining with eosin or methylene blue in alcoholic solution, or with reduced silver (from $AgNO_3$ and pyrogallol developer) caused

no apparent difference in the structure.   Fig. 1 may be interpreted as showing that the membranes are made up of granules or filaments of collodion, the pores being presumably located in the dark network surrounding the collodion.   It has not seemed possible to draw any conclusion concerning the size or number of the pores from these observations, except that the order of their size must be less than 10^{-4} cm.   The specimen photographed, like all the others examined, appeared to have about one bright spot per μ, but it is believed that the collodion filaments are much closer together than this would indicate, since the use of an 8 mm. objective did not appear to increase the size of the bright spots but did show a much greater number of hazy spots of less brightness.   The whole field, however, was so indistinct that counting was impossible, and with a 4 mm. objective it was not possible to obtain any sharp focus at all.

Validity of the Assumptions Used.

In the preceding calculations use was made of Poiseuille's law, which has been derived only for straight capillary tubes of uniform diameter. Experimentally, the rate of flow of water through these membranes has been found to be proportional to the pressure,[1, 2, 7, 8] and inversely proportional to the thickness; that is, the use of two or three membranes in place of one reduces the flow to one-half or one-third its original value.   Duclaux and Errera[8] found that the rate of flow of different liquids through similar membranes varied inversely as the viscosity of the liquids.   So far the data are in agreement with Poiseuille's law.   It might be assumed, however, that the fourth power relation would not apply to interstices between granules or filaments. Guérout[2] tested this point by measuring the rate of flow of water through sand whose grains had a mean diameter of 0.1 mm.   He calculated that the capillary spaces between the grains must be about 0.0004 sq. mm. in cross-section, while his experiments on the rate of flow gave a value, based on Poiseuille's law, of 0.0002 sq. mm.   This deviation may be partially accounted for by assuming that in the case of spherical grains the length of the capillaries ought to be greater than

[7] Bigelow, S. L., *J. Am. Chem. Soc.*, 1907, xxix, 1675.

[8] Duclaux, J., and Errera, J., *Rev. gén. colloïdes*, 1924, ii, 130; 1925, iii, 97.   In the latter paper these authors used Guérout's method to obtain figures for cellulose acetate membranes; they found $r = 1.3 \times 10^{-6}$ cm. and $n = 10^{11}$ pores per sq. cm.

the thickness of the layer of sand in the ratio of half the circumference to the diameter, or of π to 2. In any case these considerations render it probable that the values given for the mean pore radii in Table II are at least of the right order of magnitude.

The work of Bartell and Carpenter,[3] however, led them to calculate much larger pore radii for similar membranes. Their figures for the pore diameters in three membranes are 0.701, 0.934, and 1.681 μ, or, for the radii, 35, 47, and 84 $\times$ 10^{-6} cm. These figures are from 40 to 130 times as large as those in Table II. They were obtained by measurement of the pressure required to force air through a wet membrane, the relation involved being essentially the same as that used in determining surface tension by the capillary rise method. Their figures are based on the assumptions that the pores behave like capillary tubes, that they retain their original size under the pressures used (from 1.76 to 4.23 kg. per sq. cm.), that the angle of contact between water and collodion is zero, and that the surface tension of water is the same in such capillaries as it is in larger volumes. Attempts were made to apply their method to some of the membranes used in this work, but no definite flow of bubbles was obtained even at much greater pressures. This fact, together with the admitted stretching of the membranes under such pressures, renders it likely that the calculations based on Poiseuille's law are more nearly correct.

SUMMARY.

By the application of Poiseuille's law to the rate of flow of water through collodion membranes, it is calculated that the membranes used had pore radii of the order of 0.3 to 2 $\times$ 10^{-6} cm. On the same basis the number of pores per sq. cm. appears to vary from 270 $\times$ 10^{10} to 7 $\times$ 10^{10}, decreasing with increase in pore size. Reasons are given for preferring these figures for the radii to figures, 100 times as large, which were calculated by others. Microscopic examination of the membranes, with dark-field illumination, indicates that they are made up of solid granules or filaments of collodion much less than 1 $\times$ 10^{-4} cm. in thickness.

The writer wishes to acknowledge his indebtedness to Dr. John H. Northrop, under whose guidance this work was done, and to Dr. Robert T. Hance for his kind assistance in the observations with the microscope.

DURATION OF LIFE OF AN ASEPTIC DROSOPHILA CULTURE INBRED IN THE DARK FOR 230 GENERATIONS.

By JOHN H. NORTHROP.

(From the Laboratories of The Rockefeller Institute for Medical Research.)

The idea has frequently been expressed that the resistance of organisms to disease has been developed by means of natural selection, and hence that prolonged lack of exposure to infection might lead to a race lacking in resistance. Prolonged inbreeding and the absence of light have also been supposed to affect the activities of organisms. The cultures of *Drosophila* freed from microorganisms[1] by Loeb and the writer in 1916[2] have been continued since under aseptic conditions and have been kept in the dark. (The cultures have been exposed to diffuse daylight at intervals when being transferred to a new flask. In view of the marked effect of even short exposures to lower temperatures on the upper temperature limit of the organism,[3] it is possible that even this short exposure to light may be of importance.) It seemed of interest, therefore, to compare the fertility, rate of growth, and duration of life of these cultures with that of a "normal" culture when exposed to the rigors and uncertainties of a non-aseptic environment. The experiment was made under favorable conditions and also under conditions in which the duration of life was greatly shortened owing to the activities of the bacterial flora, although it is not possible to say that the insects were killed directly by the microorganisms. In neither case, however, was there any significant difference between the aseptic and the control cultures.

[1] It is naturally impossible to prove that the culture is free from all microorganisms. All that can be said is that it has not been possible to obtain a culture of microorganisms from the insects when placed in a variety of media under both aerobic and anaerobic conditions.

[2] Loeb, J., and Northrop, J. H., *J. Biol. Chem.*, 1917, xxxii, 103.

[3] Northrop, J. H., *J. Gen. Physiol.*, 1919–20, ii, 313.

EXPERIMENTAL.

The "aseptic" culture was the 230th generation of the insects freed from micro-organisms by Loeb and the writer in 1916. They had been grown since on sterilized yeast as already described.[3]

The normal culture was a strain, obtained from Dr. R. W. Glaser, which had been grown for some time on fermenting banana. They had been grown in this laboratory for 10 generations on yeast.

Yeast Medium.—280 gm. of bakers' yeast stirred up with 500 cc. of water and 15 cc. of glacial acetic acid. 25 cc. of this suspension placed in a 500 cc. flask and sufficient cotton added to absorb the excess of water. This has been found to be a very good culture medium.

TABLE I.

Comparison of Fertility, Larval Period, and Duration of Life of "Normal" Drosophila Cultures and of a 230th Generation of an "Aseptic" Culture.

Culture medium.	*Drosophila* culture.	No. of pupæ per ♀.	Days as larvæ.	Days as pupæ and imagos.
Sterile yeast cotton........	230th generation, aseptic....	6.0 ±0.5	5.0 ±0.1	27 ±0.1
	21st generation, aseptic...		6.2 ±0.1	33 ±0.2
Yeast cotton....	"Normal."...............	1.9 ±0.2	4.6 ±0.1	23 ±0.5
	"Aseptic."...............	2.8 ±0.2	4.9 ±0.1	19 ±0.3
Banana.........	"Normal."...............	0.35 ±0.01	5.1 ±0.04	7.3 ±0.2
	"Aseptic."...............	2.70 ±0.2	5.0 ±0.04	6.2 ±0.05

Sterile Yeast Medium.—Same as the preceding except that the flasks were sterilized for 1 hour at 15 lb., after being plugged with cotton.

Banana Medium.—Ripe bananas were ground in a mortar and about 25 gm. of the pulp placed in each flask. It has been repeatedly observed that banana, unless inoculated with yeast, is a very poor culture medium for these insects due apparently to the growth of bacteria. In this experiment an unpleasant odor developed in a day or so and the flies died rapidly.

Mass cultures of the two strains were made on yeast medium. The succeeding generation of flies was transferred when 1 day old to a series of 10 flasks containing the proper culture medium and placed in a constant temperature room at 25°C. for 24 hours. Each flask contained 30 to 50 flies. The parent flies were then removed and the number of males and females counted. In all cases the numbers of male and female were about equal. The number of pupæ formed were then counted at 24 hour intervals. The adult flies were transferred every 4 or 5 days to fresh flasks and the number of dead determined at daily intervals.

About 50 larvæ or imagos were in each of the 10 flasks. The average time for each individual culture was determined and these averages were then treated as individual observations. The figures given are therefore the means of these averages. This procedure, although perhaps difficult of justification, has been adopted rather than the more usual one of considering each individual insect, since the latter method gives probable errors so small that no two series of experiments ever agree within their probable errors. When the present method is used, it is possible to repeat the experiment within the error. The explanation of this is, presumably, that there are small constant differences in the culture flasks as well as in the organisms and the latter method takes these differences into consideration as well as the individual variation in the insects.

The results of the experiments are given in Table I. It is evident that there is no marked difference between the two cultures except in the number of pupæ formed per female on the banana medium. In that case the aseptic flies produced many more pupæ than the controls. This may be due either to a smaller number of eggs laid or to a greater mortality of the larvæ.

SUMMARY.

The number of pupæ formed per female, the duration of the larval period, and the duration of the pupal-imago period of a normal *Drosophila* culture and of the 230th generation of an aseptic culture which had been kept in the dark have been determined. The larval period and the pupal-imago period were found to be nearly the same for both cultures under both favorable and unfavorable conditions. There is no evidence, therefore, to show that inbreeding, absence of light, or growth in the absence of bacteria for 230 generations has had any effect either on the duration of life or on the ability of the organism to resist unfavorable bacteria.

A CONVENIENT METHOD FOR THE FORMOL TITRATION.

By JOHN H. NORTHROP.

(*From the Laboratories of The Rockefeller Institute for Medical Research.*)

The formol titration, as described by Sörensen,[1] has been most useful in the determination of amino-acids and especially in following the course of hydrolysis of proteins. The method as originally described could not be used for accurate determinations of small quantities of amino-acids owing to the difficulty of determining the exact end-point. The value obtained also depends on the point at which the titration is started. In the light of our present knowledge the success of the method depends upon the fact that in the presence of formalin the titration curve is displaced to the acid side to such an extent that the end-point of the titration is reached at a pH of about 9.0, where a sharp end-point is easily obtained, instead of at about 12.0, where the end-point is very indefinite owing to the buffer effect of the alkali itself. With a solution of a pure amino-acid or peptide, therefore, the titration gives directly the alkali equivalent of the compound. In the case of an unknown solution, however, it is necessary to select some arbitrary pH as the starting point. It so happens that practically all the amino-acids and peptides whose titration curves have been studied, have an isoelectric zone around pH 6 to 7, and that the proteins also have a flat place in the titration curve in this region although it is not the isoelectric point. In practically all solutions of proteins and their split products, therefore, it is possible to obtain a sharp end-point in this range of pH. It is, consequently, a convenient point from which to start the titration. The difficulty with the alkaline end-point is largely due to the fact that the formalin affects the color of the indicators so that it is difficult to match the standard exactly. This may be overcome by taking advantage of the property of a one color indicator which makes it possible to vary the end-point

[1] Sörensen, S. P. L., *Biochem. Z.*, 1908, vii, 45.

of the titration by varying the quantity of indicator.   That is, with a
one color indicator the color of a solution having a given quantity of
indicator at a pH near the middle of the titration curve of the indicator
will match a solution having half the quantity of indicator to which an
excess of alkali (or acid) has been added.

A method based on the above principles has been used for some years
in this laboratory and has been found very convenient and accurate,
especially for comparative results.   The solution is first titrated to
about pH 7.0, using neutral red as an indicator; formalin is then added,
and the solution titrated to about pH 9.0 with phenolphthalein.

Preparation of the Standards and Method of Titration.

The solution is diluted so as to require about 5 cc. of 0.01 M NaOH for 5 cc.

Neutral Red Standard.—5 cc. of the solution is pipetted into a test-tube; 1 cc.
of 0.05 M sodium phosphate and 1 drop of dilute neutral red are added.   The
solution is then titrated with either acid or alkali until it is at the point of sharp
color change of the indicator.   (Owing to the salt and protein errors of the indi-
cator, the pH value of this point varies somewhat with different solutions, but is
usually about pH 7.   The exact figure can be obtained for any solution by deter-
mining the pH electrometrically.)

Alkaline Standard.—5 cc. of solution, 1 drop of neutral red, 1 drop of 0.1 per
cent phenolphthalein, and 1 cc. of 40 per cent formaldehyde solution are placed
in a test-tube and 0.01 M NaOH added until the maximum color is developed
(an excess of alkali does not interfere).   This gives automatically a pH of
about 8.5.

Titration of the Solution.—1 drop of neutral red is added to 5 cc. of the solution
and the solution titrated to match the "neutral" standard.   This titration is
made roughly with strong alkali and then completed with 0.01 M NaOH in order
to avoid increasing the volume.   In most cases this end-point can be deter-
mined with an accuracy of 0.05 cc.   1 cc. of formalin is then added and *3 drops*
of 0.2 per cent phenolphthalein, and the solution titrated with 0.01 M NaOH
to match the "alkaline" standard.   This end-point can also usually be determined
to 0.05 cc. even in colored solutions, owing to the fact that an exact match with
the standard can be obtained.   The amount of alkali required to bring the solu-
tion from the "neutral" standard to the "alkaline" standard is the titration
figure, and, in the case of amino-acids and simple dipeptides at any rate, agrees
quite closely with the alkali equivalent of the substance as determined by electro-
metric titration.

Formaldehyde Blank.—The blank for the formaldehyde is obtained by carrying
out the titration as above with water instead of the amino-acid solution.   The
presence of any other acid or base in the solution, and especially of substances

acting as buffers in this range, of course causes an error.   Comparative values for the increase in the alkali-binding power can still be obtained.   Phosphates can be removed by precipitation with barium, and carbonates may be removed by acidifying and boiling or aerating.   In the case of solutions of pure amino-acids or peptides the preliminary titration to the neutral standard can be omitted, as can also be done when the increase in titration of the same solution is being followed.

Significance of the Titration Value Obtained in This Way.

As stated above, the figure for the alkali-binding capacity agrees in the case of the amino-acids with the total alkali-binding capacity of the amino-acid.   If the alkali reacts with the free carboxyl groups then the figure gives the normality of the carboxyl groups present. If, however, the amino-acids are present in solution as *Zwitterionen*, as Bjerrum[2] suggests, and as there is good reason to believe, then the figure obtained is the amino group equivalent.

[2] Bjerrum, N., *Z. physik. Chem.*, 1923, civ, 147.

A DISCUSSION OF RECENT STUDIES ON THE METABOLISM OF NORMAL AND MALIGNANT CELLS.

By JAMES A. HAWKINS.

(From the Laboratories of The Rockefeller Institute for Medical Research.)

The importance of lactic acid as a product in the carbohydrate metabolism of living tissue is well recognized. Salkowski (1) in 1890 stated that muscle produces lactic acid not because it is dying but because it is living and produces it only during life. Fletcher and Hopkins (2) have shown that under anaerobic conditions lactic acid is spontaneously developed in freshly excised resting muscle, but only in very small quantities in the air and not at all in the presence of pure oxygen. Hill and Meyerhof (3) consider that glycolysis is the chemical process involving the whole (or nearly the whole) of the energy liberated in the initial phases of contraction of muscle. Levene and Meyer (4) found that the leucocytes of rats are able to diminish markedly the reducing power of a glucose solution and that kidney tissue of rabbits also has this property but to a lesser extent. They were able to isolate paralactic acid as the zinc salt from the solution, and thus definitely established for the first time the fact that lactic acid is an intermediate product of glycolysis in living tissues.

Warburg (5) and his associates in a series of studies have reported on the metabolism of malignant tissues as compared to that of normal tissues. They found that the respiration of the Flexner-Jobling carcinoma was considerably less than that of normal tissue. Further investigation showed that even this small amount of respiration in tumor tissue suspended in Ringer's solution was inhibited by the addition of glucose. The lactic acid produced by glycolysis was sufficient to stop respiration. The respiration of normal tissue was not influenced by the addition of glucose and only minute amounts of lactic acid were formed.

Warburg found that malignant tumor cells produce three to four times as much lactic acid per molecule of oxygen consumed as do

benign tumors, and that a 3 to 5 day old chick embryo in the absence of oxygen produces lactic acid at almost the same rate as malignant or benign tumor tissues but that in the presence of oxygen normal respiration takes place with the formation of very little lactic acid.

Negelein (6) recently has shown that rat embryos, suspended in inactivated horse serum, produce large amounts of lactic acid in the absence of oxygen and in the earlier stages of development produce some lactic acid in the presence of oxygen. The amnion and chorion of these embryos also produce large amounts of lactic acid in the absence of oxygen. He found that the glycolytic activity of these tissues was at a maximum in the earlier stages of development of the embryo when the growth of the embryo is most rapid.

From these results, Warburg divides tissues into four types on the basis of metabolism: normal resting tissue with a high respiratory rate and slight anaerobic glycolysis; embryonic tissue with a high respiratory rate and high anaerobic but low aerobic glycolysis; malignant tumor tissue with a low respiration and high anaerobic and aerobic glycolysis; benign tumor tissue with the same type of metabolism as malignant tissue but with a lower aerobic glycolysis. He classifies tissues on the basis of their aerobic metabolism, as malignant if the glycolysis-respiration ratio is 3, as benign tissue if the ratio is 1, and as normal growing tissues if the respiration suffices to bring about the disappearance of glycolysis products.

Using Warburg's technique and working with the same types of tissue as used by him we obtained results essentially the same as his and the differences between the groups were clear-cut (7). However, when other tissues were investigated the groups became less definite. For example, rat spleen, embryonic skin, and the wall of a pregnant uterus had the typical embryonic type of metabolism. Rat placenta grouped according to this scheme fell within the malignant group. The transplantable Chicken Tumor 1 could be grouped with the transplantable rat carcinoma, but Chicken Tumor 9 showed considerable variation. The latter tumor, grouped on the aerobic glycolysis respiration ratio, in the majority of instances had about the same range as Chicken Tumor 1; but a rapidly retrogressing tumor composed almost entirely of reactive tissue frequently gave as high a ratio as the progressive tumors made up entirely of intact tumor cells.

The results with the spontaneous tumors of mice were even more varied. Grouped on the basis of their types of metabolism the majority behaved as embryonic tissue, some fell in the group of benign tumors, and a very few could be classed as malignant. Histologically, all of these tumors are typically malignant. Classified on the basis of
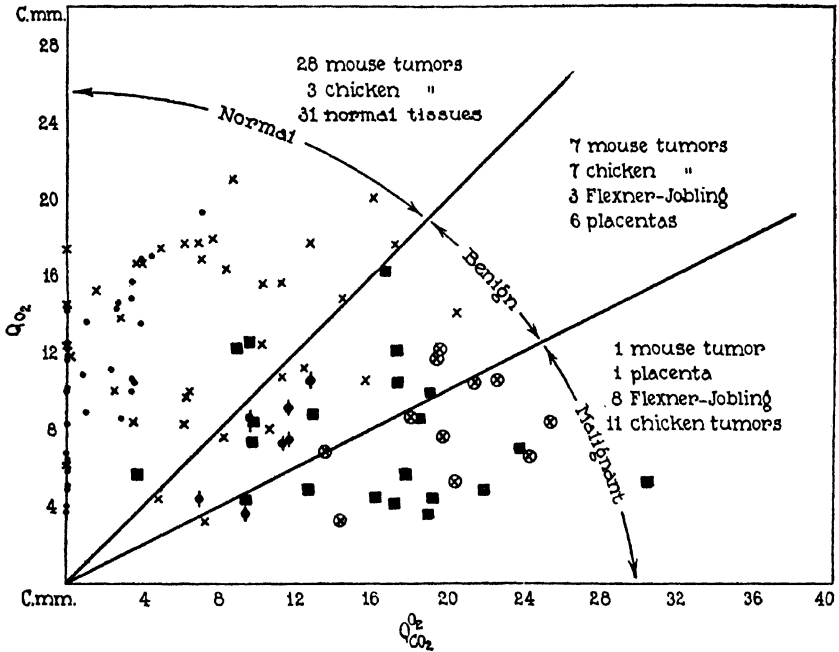
FIG. 1. The oxygen consumption in c.mm. per mg. of dried tissue per hour is plotted as ordinates and the aerobic glycolysis in c. mm. per mg. of dried tissue per hour as abscissæ. Each point (●) represents an individual observation on normal tissues, each symbol ◆ on placentas. Each of the other symbols represents an individual observation on malignant tissues: × mouse tumors, ■ chicken tumors, ⊗ Flexner-Jobling tumors. The areas normal, benign, and malignant indicate the areas bounded by the aerobic glycolysis-respiration ratios 0 and 1, 1 and 2, and 2 and over respectively.

their biological behavior they are in all essentials similar to malignant tumors as they occur in man. The results are shown graphically in Fig. 1.

The aerobic glycolytic activity seems to bear no relationship to

growth rate. A very slowly growing or retrogressing chicken tumor frequently gave as high an aerobic glycolysis-respiration ratio as a rapidly growing tumor. The rapidly progressing spontaneous mouse tumors often gave a low ratio while the slower growing ones in some instances gave a relatively high ratio.

If we examine these results on another basis, *i.e.* that of energy production, we find that the total aerobic energy per mg. of dried tissue falls within the same range for all the tissues.

The amount of energy obtained per mg. of tissue may be calculated from the following data by Slater (8):

Heat of combustion of 1 gm. of glycogen monohydrate in dilute solution... 3836 cals.
Heat of combustion of 1 gm. of lactic acid in dilute solution .. 3601 "
Heat available due to the formation of 1 gm. of lactic acid from 1 gm. of glycogen................................. 235 "
Heat of neutralization of 1 gm. of lactic acid by alkali protein as determined by Meyerhof.......................... 138 "
Total heat liberated per gm. of lactic acid formed.......... 373 "

Therefore every c.mm. of CO_2 formed by the combustion of glycogen represents 5.09×10^{-3} cals., and every c.mm. of CO_2 obtained by the splitting of glycogen to lactic acid represents 1.50×10^{-3} cals. Consequently the total aerobic energy per mg. of dried tissue per hour is equal to the c.mm. of CO_2 obtained by respiration multiplied by 5.09×10^{-3} cals. plus the c.mm. of CO_2 obtained by glycolysis multiplied by 1.50×10^{-3} cals.

From the results shown in Fig. 2 it is apparent that the energy per mg. of dried tissue is approximately the same whether this energy is obtained entirely by the combustion of glucose or by the combustion of glucose and the splitting of glucose to lactic acid. So tissue with a low respiratory rate and high glycolytic activity obtains practically the same energy as tissue with high respiration and no glycolysis. Fig. 3 shows the results obtained by using the published data of Warburg, Posener, and Negelein (9) to calculate the energy per mg. of dried tissue per hour. There is a close agreement between his results and ours.

That the results obtained per mg. of dried tissue for normal and for malignant tissues are comparable would seem to be borne out by the
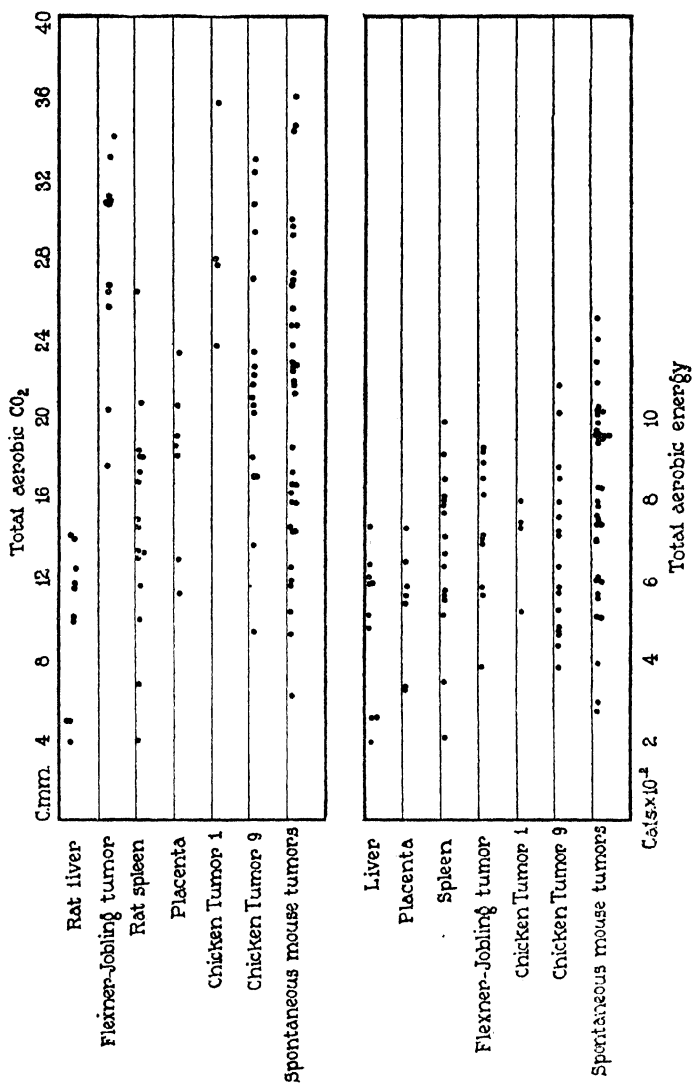
FIG. 2. Each point in the upper chart represents an individual determination of the total aerobic carbon dioxide in c.mn. per mg. of dried tissue per hour produced by the various tissues.

Each point in the lower chart represents an individual determination of the total aerobic energy in cals. $\times 10^{-2}$ per mg. of dried tissue per hour produced by the various tissues.
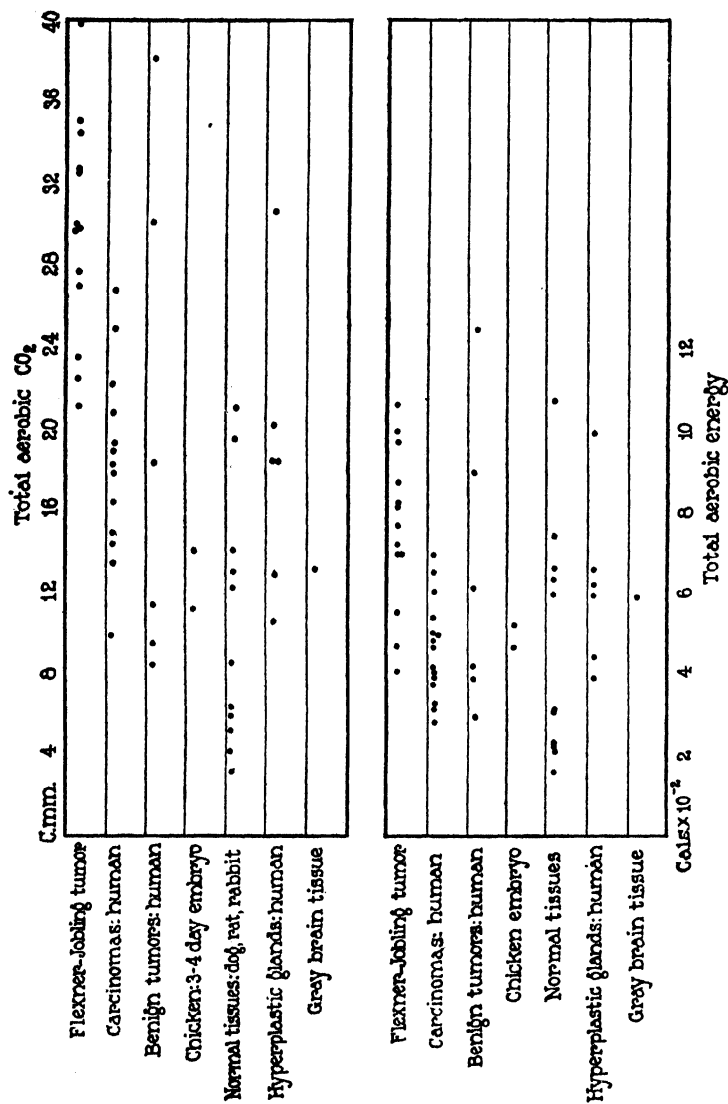
FIG. 3. For explanation see Fig. 2. These determinations were taken from the published data of Warburg.
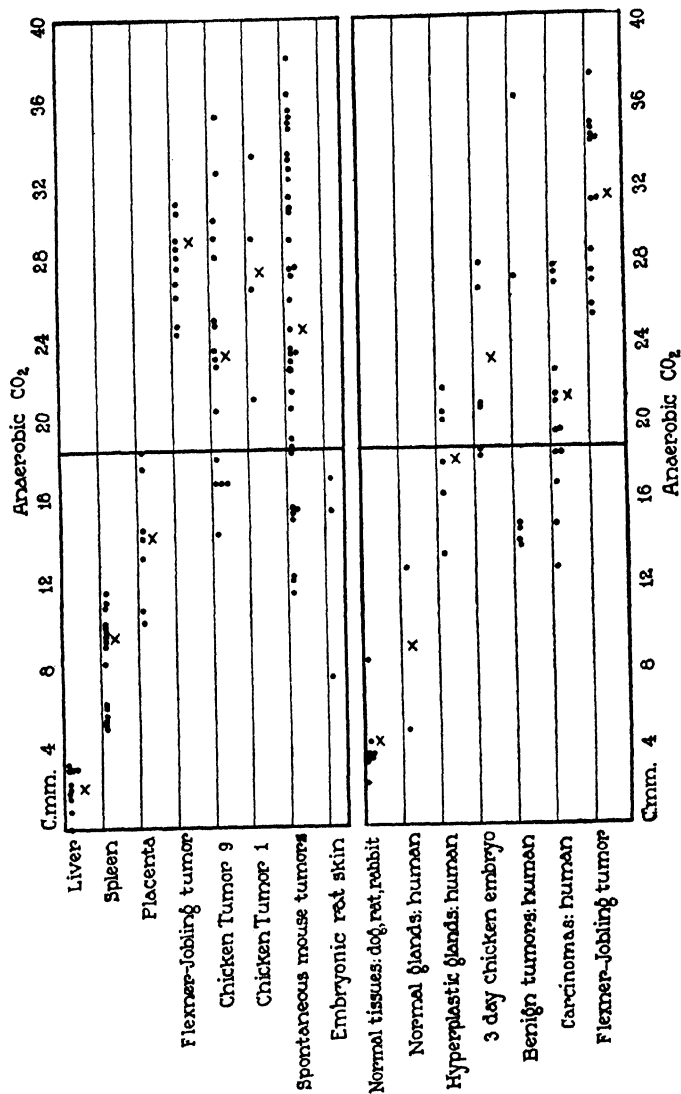
FIG. 4. Each point represents an individual determination of the anaerobic glycolysis expressed in c.mm. of carbon dioxide per mg. of dried tissue per hour. The crosses represent the average for each class of tissue. The heavy vertical line is drawn to divide normal and malignant groups. The determinations in the lower chart were taken from the published data of Warburg.

findings of Lewis and Lewis (10) that there is a striking similarity between the growth of normal tissues of embryo chicks in salt solutions, nutrient agar, and plasma, and that pictured by Lambert and Hanes for the growth of rat and mouse sarcoma and carcinoma in plasma.

If we turn now to the anaerobic conditions where glycolysis is favored, we find that all tissues have some glycolytic activity. If the tissues are arranged in groups we find that each group has a different amount of glycolysis as expressed in c.mm. of CO_2 per mg. of dried tissue per hour. Normal tissues produce very little lactic acid, embryonic tissues and placenta slightly more, and malignant tumor tissue still more. If the maximum amount of CO_2 produced by placenta is taken as a limit for normal tissue it is found that malignant tissues as a rule group themselves above this point.

In Fig. 4 are shown the c.mm. of CO_2 produced by various tissues by anaerobic glycolysis. The normal tissues liver, spleen, embryonic rat skin, and placentas, all produce less than 19 c.mm. of CO_2 per mg. of dried tissue per hour. In the Chicken Tumor 9 group, five tumors produce less than 19 c.mm. of CO_2 but four of these were tissues taken from retrogressing tumors, showing practically no intact tumor cells, the mass being composed mostly of reactive tissue. Eight of the spontaneous mouse tumors produced less than 19 c.mm. of CO_2 and seven of these were tumors on which accurate measurements for 4 weeks showed very little growth.

In the lower half of the chart are given the published data of Warburg. The results are not very clear-cut, as five tissues which he classified as benign tumors are above the line while five tumors classified as malignant are below; but, if we used his corrected results, four of these five would be above the line. The 3 to 5 day old chick embryos are on the border line or above, but the growth rate of these could be compared favorably with that of malignant tissue.

<center>DISCUSSION.</center>

It would seem from these results and from the findings of Negelein on the glycolytic activity of young rat embryos and chorion, that glycolytic activity of a tissue is a function of its growth rate. On this basis the malignant tissues in most instances having a more rapid growth rate than normal tissues fall in a group by themselves, and are only approached by the young embryonic tissues.

We have extended the findings of Warburg and differ from him in the interpretation of the results. We believe that the anaerobic glycolytic activity of tissues is a function of their growth rate, and that from this activity a classification of tissues may be made corresponding to their biological groupings much more closely than the classification of tissues from the aerobic glycolysis-respiration ratio as used by Warburg.

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PHYSIOLOGICAL ONTOGENY.

A. Chicken Embryos.

X. The Temperature Characteristic for the Contraction Rate of Isolated Fragments of Embryonic Heart Muscle.

By HENRY A. MURRAY, Jr.

With the Assistance of Yetta Porosowsky.

(*From the Hospital of The Rockefeller Institute for Medical Research.*)

It is our purpose to characterize heart muscle structurally and functionally in various ways at successive ages. In view of Crozier's[1] recent work which suggested that the quantitatively determined effect of temperature on the velocity of biological processes may serve to identify the nature of the underlying reaction, it was deemed of interest to determine the temperature characteristic, and to discover whether, in the case of rhythmically contracting fragments of heart muscle, the effect of temperature upon the rate of contraction might not be functional to the age of the embryo and the location in the heart from which the section was removed. This particular investigation was facilitated by the fact that during the course of other experiments we had occasion to make numerous cultures of chick embryonic heart muscle sections in clotted plasma, which were found to contract rhythmically for many days without external stimulation and thus to provide almost ideal material ready at hand for the present purpose.

Following Arrhenius, who first pointed out that a linear relationship exists between the logarithm of the velocity of a chemical reaction and the reciprocal of the absolute temperature, biologists showed that the velocities of numerous physiological processes were similarly affected by temperature, a fact which gave presumptive evidence of the chemical nature of such phenomena.

[1] Crozier, W. J., Numerous papers in *J. Gen. Physiol.*, 1923–25, especially *J. Gen. Physiol.*, 1924–25, vii, 189.

More recently Crozier has collected from the literature the data on temperature effects that were capable of mathematical treatment. He has used the equation of Arrhenius in the form:

$$\frac{K_2}{K_1} = e^{\frac{\mu}{2}\left(\frac{1}{T_1} - \frac{1}{T_2}\right)},$$

where K_1 is the velocity at absolute temperature T_1, and K_2 the velocity at T_2, and $\frac{\mu}{2}$ is the *critical thermal increment* (μ) divided by the gas constant (2). In the case in which $T_2 = T_1 + 10$ then $\frac{K_2}{K_1} =$ the temperature coefficient (Q_{10}) for that temperature interval, as commonly employed by the chemist. To bring into relief the value μ the above equation may be written:

$$\mu = 4.61 \frac{\log K_2 - \log K_1}{\frac{1}{T_1} - \frac{1}{T_2}}$$

Investigators entertained the hope that a knowledge of the effect of temperature upon the rates of biological reactions would elucidate the nature of the slowest in the complex of reactions which made up the phenomenon under observation. Very little was discovered when the temperature coefficient was used as an index, except to separate the chemical from the physical phenomena, as the former were found to have lower or negative temperature coefficients. The emphasis of Crozier upon the value of μ instead of Q_{10} seems to depend upon three advantages in its use: (1) a greater range of values as empirically determined; for, whereas Q_{10} for chemical reactions usually varies between 2.0 and 4.0, μ has a range between 4,000 and 35,000; (2) the fact that in some cases at a certain point in the temperature scale an abrupt change in the value of μ may occur, indicating a shift to a new limiting reaction, which is masked if the usual method of obtaining Q_{10} is used. (3) The value for Q_{10} is not constant but changes continuously with the temperature; whereas this is not true for μ. The chief purposes of obtaining the temperature increment for a biological process are (1) as a criterion for exact empirical classification, and (2) to discover the chief underlying reactions by a comparison with temperature characteristics of simple known chemical reactions.

Crozier has brought forward evidence to show that the values grouped around 11,500 depend upon a reaction catalyzed by hydroxyl ions, whereas those between 16,000 and 17,000 involve some oxidation-reduction system. These speculations are partly based upon the work of Rice and others who have attempted on theoretical grounds to give μ some meaning. It has been found that diverse reactions yield the same temperature characteristic provided they have the same catalyst, and conversely that the same reaction exhibits different temperature characteristics when activated by different catalysts. Finally it seems that μ is specific for a reaction and is independent of the amount of catalyst.

Technique.

Experiments were done in an electrically heated box, which contained the culture plate on the stage of the microscope. The temperature was raised and lowered by closing and opening an outlet on the top of the box. The fragments cut from the auricles and ventricles of chick hearts of different ages were implanted in plasma on the under surface of the mica cover glass of a Gabritschewski dish according to our usual tissue culture technique. A thermometer resting upon the mica immediately above the fragment under observation registered the temperature. The contractions observed through the microscope were counted with the aid of a stop-watch or in other ways.

Observations were made between 32° and 40°C. since there is danger of damaging the contraction mechanism outside that range. It was found that the heart fragment was more sensitive than the thermometer, and that on account of the lag in the latter, the temperature had to be changed slowly. It was the practice to lower and raise the temperature through the entire scale. If the rate of contraction as it changed with temperature did not travel the same course on both its journeys, i.e. up and down, and did not return to the same point from which it started, the experiment was discarded; if it seemed to be satisfactory in these respects, however, the procedure was repeated once, twice, or even three times, the experiment covering 2 to 5 hours of continuous observation.
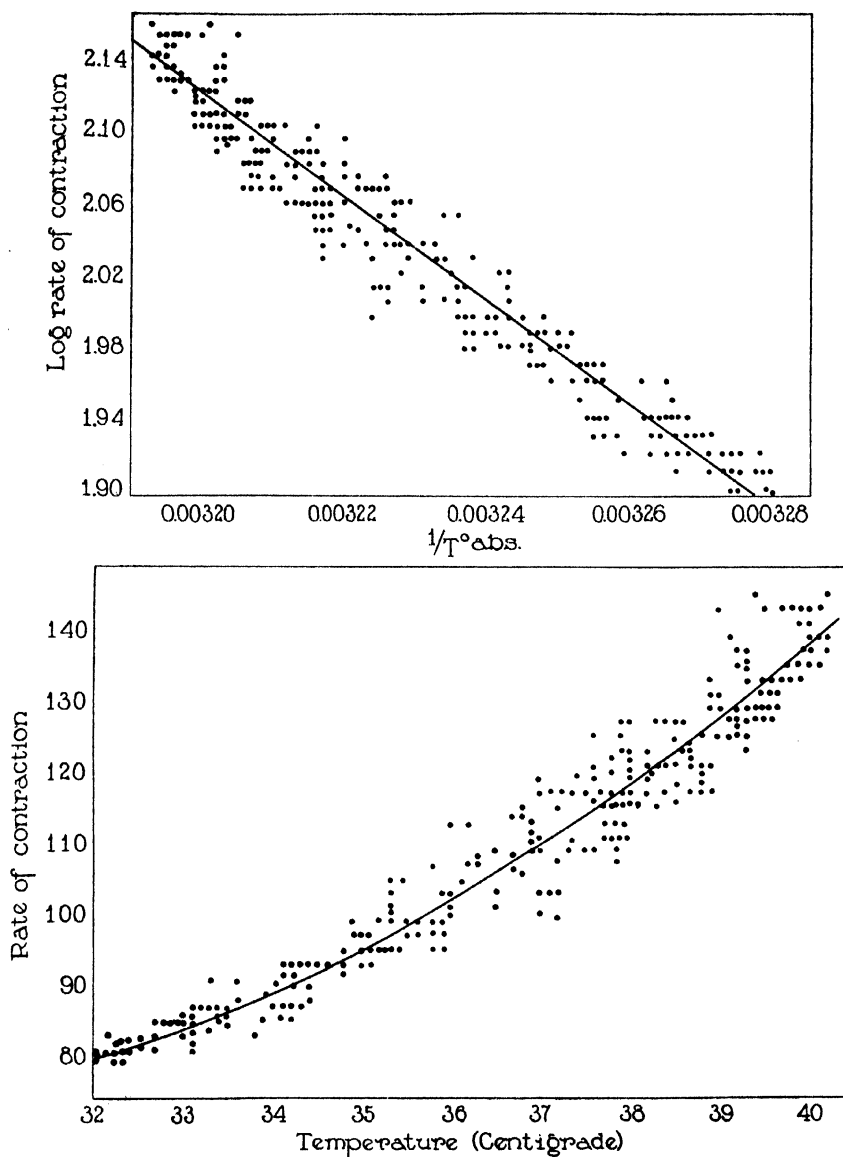
FIG. 1. The contraction rate of an auricular fragment from an 8 day embryo compared with the temperature in two ways.

RESULTS.

After trying many ways of equating velocity with temperature, the conclusion was reached that the Arrhenius equation was the only one that would hold in the majority of cases covering the entire range of contraction rate.

In general, at 38°C. fragments from the auricles beat two to six times faster than those from the ventricles. The former showed variations from 60 to 180, approximately, whereas the latter's range was limited between about 25 and 40 beats per minute. When the logarithm

TABLE I.

Temperature Characteristic (μ) for Auricular and Ventricular Fragments of Embryonic Heart Muscle.

Age.	Auricle.				Ventricle.			
	No. of experiments.	Average rate of contractions.*	Temperature characteristic.	Standard deviation. ±	No. of experiments.	Average rate of contraction.*	Temperature characteristic.	Standard deviation. ±
days								
3	1	38	23,500					
4	1	80	21,300		1	37	10,900	
5	1	156	13,400		1	30	17,100	
6	3	121	13,000	4,000	1	27	8,300	
7	1	76	14,300		1	35	14,400	
8	8	113	14,800	1,000	2	37	17,300	1,000
14	1	182	13,800					
15	1	130	10,100					
16	1	125	8,300					
17	1	162	9,200					

*Taken at 38°C.

of the rate was plotted against the reciprocal of the absolute temperature there was often a slight curve, either convex upward or downward, but as the number of examples of each type balanced, these small divergences were not considered significant and the slope of the best straight line determined the value of μ (Fig. 1). As care was taken to discard all experiments in which the contracting fragment did not repeat its performance at least once over the entire scale of recorded temperatures, and inasmuch as in some cases 200 or more observations were made of one piece, some confidence was felt in the accuracy of the results (Table 1). From an examination of the figures and the

table it may be observed that there is the greatest irregularity in the values obtained (Figs. 2 and 3). For instance in the eight 8 day auricular sections examined, the value of μ varied from 9,000 it 18,000,
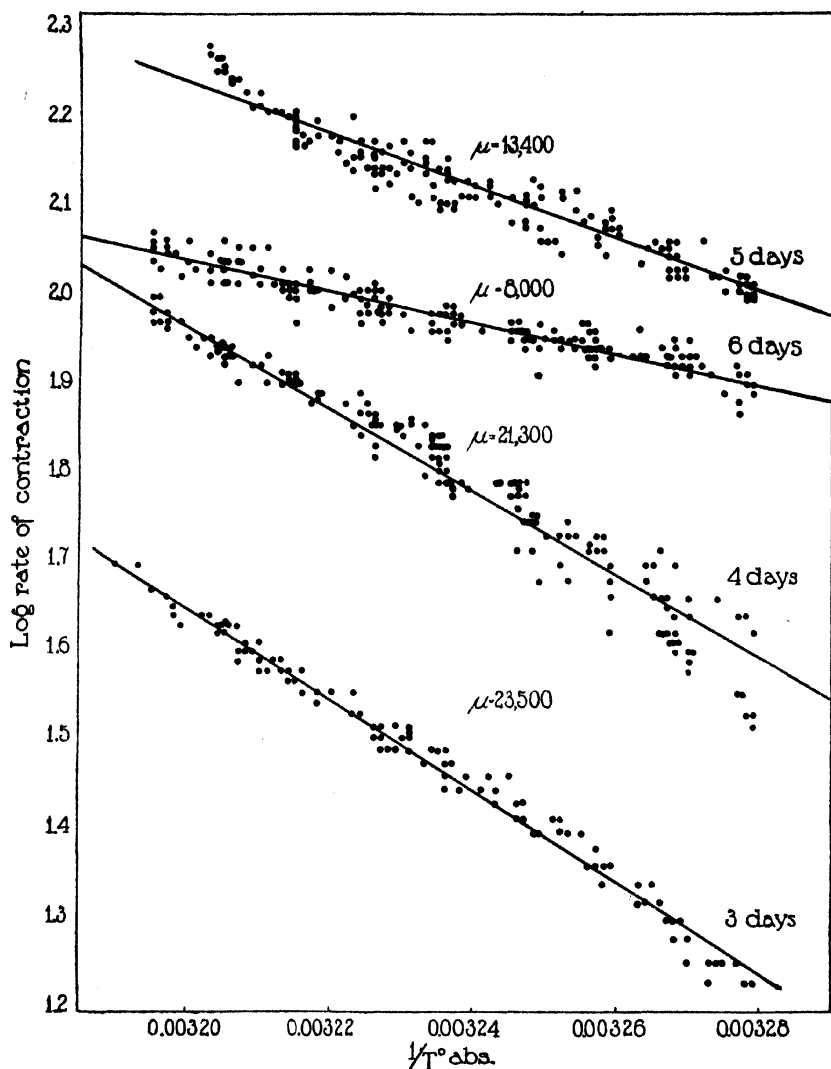
FIG. 2. The contraction rate of auricular fragments from embryos of 3, 4, 5, and 6 days of incubation respectively.

and the rate of contraction at 38° from 51 to 167. No significant correlations could be made between μ (the critical thermal increment)
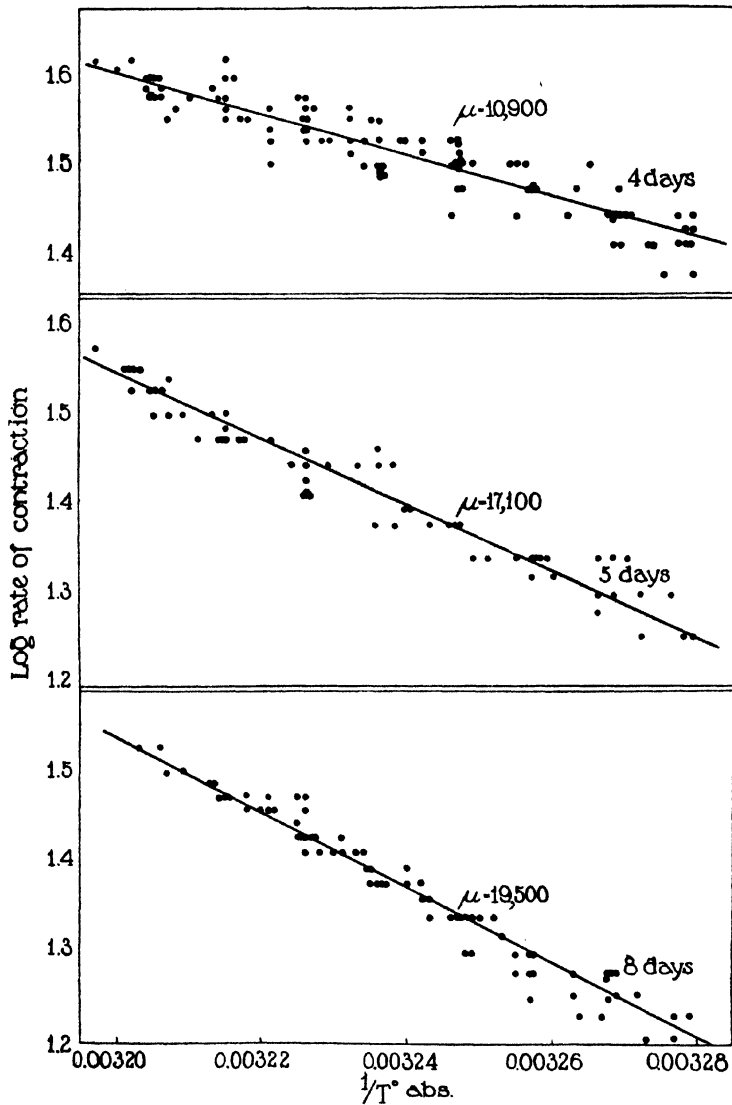
FIG. 3. The contraction rate of ventricular fragments from embryos of 4, 5, and 8 days of incubation respectively.

and any other function, such as the age, the original location of the cardiac fragment, or the contraction rate at 38°C. Comparing the average figures for auricular fragments there seems to be a slight tendency towards a diminution in temperature effect with age. By extending the curve down to 30°C. a theoretical figure was obtained for the contraction rate, and from this $\dfrac{K_{40°}}{K_{30°}} = Q_{10} =$ temperature coefficient. The temperature coefficients calculated in this way were found to vary between 1.5 and 4.3.

These facts might lead one to question the significance of the temperature characteristic, inasmuch as pieces from the same heart presumably exhibiting similar chemical reactions and activated by similar enzymes seem to show no tendency to uniformity. The averages range themselves around 14,000 $\pm$, but only three experiments out of the lot actually gave this value; a value, moreover, which is not commonly encountered for biological processes.

Tissue dislocated from the patterning forces of the organism has thus been found to behave in an irregular fashion. Possibly the regularity of the temperature effect as empirically determined upon certain functions *in vivo* depends to a greater extent than is acknowledged upon the integrity of the organization and the interrelationships of the parts of the whole, and less upon one particular chemical reaction or catalyst.

CONCLUSIONS.

1. The Arrhenius equation giving the relationship between the velocity of chemical reaction and temperature, was found suitable for the special case of the contraction rate of embryonic heart muscle fragments.

2. There was no constancy in the values of μ for the rate of contraction in culture, nor was the scattering evenly distributed around certain (more than one) points.

3. There seemed to be no correlation between μ and other functions such as the contraction rate, the site from which the piece was removed, the age of the embryo, etc.

PHYSIOLOGICAL ONTOGENY.

A. CHICKEN EMBRYOS.

XI. THE pH, CHLORIDE, CARBONIC ACID, AND PROTEIN CONCENTRATIONS IN THE TISSUES AS FUNCTIONS OF AGE.

BY HENRY A. MURRAY, JR.

WITH THE ASSISTANCE OF YETTA POROSOWSKY.

A previous paper (1) reported preliminary analyses of the changes with age in the chief organic constituents (carbohydrate, protein, and fat) of the chicken embryo. The present study concerns itself rather with the physicochemical conditions of the tissues, at least in so far as these can be defined by a knowledge of the concentration of protein, chloride, bicarbonate, and hydrogen ions. The notion was entertained that their simultaneous representation with time variable would provide a simple description of the changing internal environment. It is assumed that the reacting substances and their milieu are mutually interrelated, so that a knowledge of the latter will bear upon the vital activity of the former. It is understood that the present study is statistical in so far as the analyses give artificial values which are to be taken quite arbitrarily to represent some average, the significance of which is not rationally to be deduced. Moreover, in the present state of our ignorance in respect to the physicochemical organization within the functioning cell there is considerable doubt as to the value of these analytic results for the representation of physiological conditions. Particularly is it necessary to examine with suspension of judgment such questions as the normal average range of hydrogen ion concentration in the tissues. Experimental findings and theoretical considerations show that the pH as well as the concentration of other ions varies in different parts of the body, of an organ, and of any one cell. In a metazoan, there-

fore, any figure obtained for the whole organism would be an average value, not necessarily corresponding to the actual conditions at any one locus. It is just these average values, however, which we at present seek to find so that correlations may be made between various chemical constituents in terms of the age of the organism.

Methods.

pH.

There is uncertainty about any described technical procedure, and, but for the relevancy of the H ion concentration in defining important relationships, we should not have undertaken to determine it. No procedure has as yet been developed which does not require an assumption of the constancy of a number of theoretically variable factors. In so much as we seek the changes in pH with age rather than the actual value at any one time, an unknown error, if relatively constant, would not vitiate a conclusion as to the direction of change which the results might seem to indicate.

For multicellular organisms four chief methods for the determination of average pH have been described, two of which are: (a) injection of vital dyes (2) and (b) microscopic observation of the color of an indicator within cells after small fragments of tissue bathed in dye solution have been squeezed sufficiently to rupture cell membranes and then released so as to allow the indicator to flow back into the interior with some of the expressed cytoplasm (3). These methods have given results of interest for other studies, but as the values obtained are purely local and vary from apparent values of pH 3.0 for intracellular granules in some cells to values higher than in blood for bone and cartilage, one is not able to arrive at an average pH figure for the whole organism. Moreover, one is unable to predicate (1) whether the dye itself affects the equilibrium which it is supposed to measure, (2) what corrections are necessary for protein and other factors within the cell—for these cannot be minimized by dilution,—and (3) to what degree exposure of the surface of the section, organ, or body to oil or air affects the color *in vivo*.

The two other important methods depend upon: (c) potentiometer readings at the moment of the thawing of tissues which have been frozen, triturated, and introduced in the form of solid masses into the hydrogen electrode cell (3), and (d) gross compression of tissues with colorimetric determination of the expressed fluid (3, 4).

When frozen tissue is placed within an electrode cell a progressive change in pH is recorded as the temperature is gradually raised. It seems that in certain parts of the temperature curve obtained the pH changes are rather sudden, and

Vlès, who has worked with this method, is inclined to attribute these sudden shifts to dissociations involving different radicles of the cell proteins. Vlès' results showed great variations under fixed conditions. The technique is somewhat elaborate, and as we do not know what changes occur upon freezing and thawing and at what point one may consider the reading obtained to represent the true pH of the tissue fluids, we turned to method (*d*) or some modification of it as more suitable for our purposes. Michaelis, and Vlès who have used this method, made no satisfactory provision for the immediate escape of CO_2 from tissue surfaces exposed to air. In our experiments we covered the material with oil to minimize this error. Moreover, there is something to be said in favor of our method from the standpoint of simplicity. The only special piece of apparatus
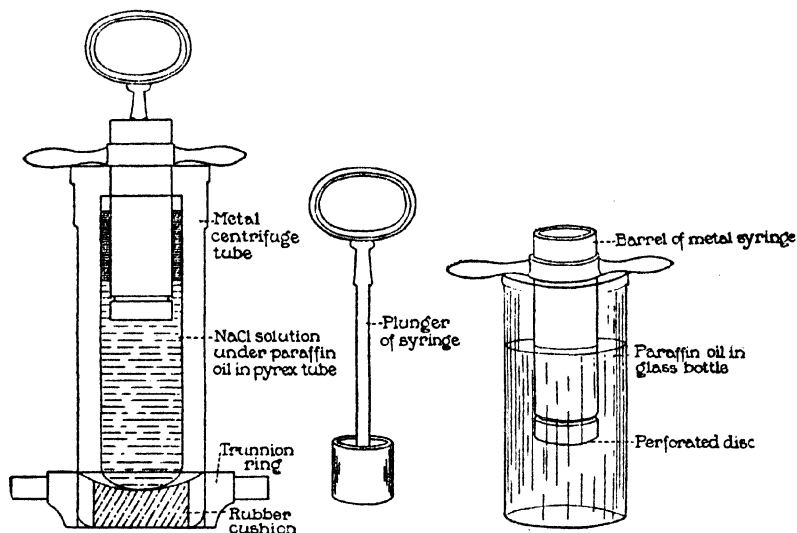
FIG. 1. The apparatus employed for determining the average pH of the tissues of chicken embryos.

required is a metal syringe for the compression of embryonic tissue, with a perforated bottom to allow for the filtration of the expressed fluids. The syringe has a diameter of 15 mm., which is a convenient size for embryos up to 15 days of age. The barrel, capped with a perforated disc, is half submerged in paraffin oil in a small glass bottle (Fig. 1). The egg is opened and as expeditiously as possible the membranes are cut, the embryo lifted out with forceps, and dropped into the oil-containing syringe. The piston is then inserted and the syringe inverted to expel the air. It is immediately introduced into the pyrex tube *B* which contains the indicator dissolved in sodium chloride under oil at such a level that the perforated surface is well below the oil in the tube. The arms of the syringe engage

the edge of the metal centrifuge tube so that all possible force can be applied downward upon the handle of the piston. Juice, with disintegrated formed elements, passes through the sieve into the NaCl solution. 2 minutes of centrifuging throws down the red blood cells and formed elements, leaving the clear solution of tissue juice and indicator in the supernatant saline. The pH of this fluid is read by comparison with colorimetric standards. The method is not unlike Hawkins' procedure for blood (5). For making the standards the bicolor principle was used. Each pH standard value is obtained by placing 2 tubes in a comparator block in line with a blank tube containing dissolved tissue juice of appropriate concentration without dye. One tube containing a certain concen-
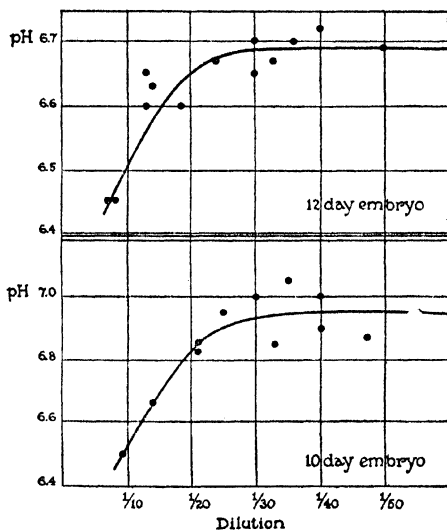
FIG. 2. The effect of dilution (as measured by the amount of formed elements thrown down in centrifuging) upon the value of the pH of tissues.

tration of the dye in its alkaline form and the other a certain concentration of the same dye in its acid form. The tubes were selected from a graded series of each form so as to provide by their combination the color shade corresponding to the desired pH. For the preparation of the standards the technique recently perfected by Hastings and Sendroy has been followed in detail (6).

As has previously been shown for blood, considerable dilution is necessary before constant readings practically independent of further dilution could be made. In the case of tissues it was found that dilutions of 1/30 to 1/70 (as measured by the amount of formed elements thrown down in centrifuging, which is equivalent to about

1/25 to 1/60 in terms of the total volume of extract) gave satisfactory results; or in other words that with 20 cc. of saline the total amount of tissue extract, *i.e.* formed elements + fluid used must lie between 0.33 and 0.80 gm. approximately (Fig. 2).

Bicarbonate and Chloride.

As Van Slyke and others have shown for blood, it would seem that the Cl ion and the HCO_3 ion were perhaps quantitatively and physiologically the most important anions in the body. The method devised by Van Slyke for the estimation of total carbon dioxide in solid carbonates (7), with a few modifications kindly suggested by Dr. Van Slyke himself was used.

Embryos were rapidly separated from their membranes and dropped into 3 cc. of 1.0 N NaOH solution in a weighing bottle. Exposed to the air they were expeditiously and thoroughly cut up with fine scissors, weighed, and then transferred with 10 cc. of water to a 250 cc. suction flask. In the flask was then inserted a tube containing 10 cc. of approximately 0.05 N $Ba(OH)_2$ solution. In the tube was put a small glass rod, which, when the flask was gently rotated, served to stir effectively the contents of the tube. The flask was then evacuated and after standing more than an hour, 5 cc. of 4.0 N lactic acid was allowed to flow into the embryonic extract, so that the NaOH was more than neutralized and because of the evacuated condition of the flask there was a ready evolution of carbon dioxide which combined with the barium hydroxide and was precipitated as barium carbonate. After a 24 hour interval in which the bottle was shaken frequently the barium hydroxide solution in the tube was filtered through a Gooch crucible, washed with 40 cc. of water, and titrated with HCl. Two controls were done with each set of eight determinations and the difference between the titration results for each embryo and the average value for the two controls indicated the amount of carbonic acid given off by the embryonic extract.

The chlorides were estimated by Van Slyke's recently simplified method for tissues, whereby the entire analysis is carried out in one flask (8).

Protein.

The nitrogen estimations reported in a previous paper (1) were used for our calculations. The results multiplied by 6.25 were assumed to be equal to the concentration of protein. A complicating factor is the growth of feathers during the last week of incubation. The feathers, like bone, may be said to have no real part in the dynamic activity of the body, and thus their inclusion in the analysis of

the whole embryo would tend to distort the composite picture by which one might seek to represent the physicochemical pattern of the functioning tissues. The weight of the feathers was determined from the time that they attain measurable mass, *i.e.* from the 13th day of incubation onwards, and then corrections in the total protein

TABLE I.

The Concentrations of Certain Chemical Constituents of the Tissues of Chicken Embryos as Functions of Age.

Age.	pH		Chloride.				Total carbonic acid.				Feathers, per cent of total weight.	Protein, gm. per 100 gm. H₂O.*	Fat, gm. per 100 gm. H₂O.*
	pH	Standard error. ±	Per cent of total solid.	Standard error. ±	Gm. per 100 gm. H₂O.	Millimols.	Per cent of total weight.	Standard error. ±	Gm. per 100 gm. H₂O.	Millimols.			
days													
5												3.95	0.81
6			4.82	0.30	0.291	82.1						4.17	0.87
7			4.76	0.08	0.289	81.5	0.062	0.004	0.066	15.0		4.38	0 95
8	7.00	0	4.48	0.12	0.281	79.3	0.066	0.007	0.070	15.9		4.67	1.03
9	6.96	0.03	4.27	0.10	0.285	80.4	0.074	0.004	0.079	18.0		4.88	1.11
10	6.95	0.03	3.73	0.06	0.272	76.7	0.064	0.002	0.069	15.7		5.31	1.24
11	6.91	0.03	3.10	0	0.265	74.8	0.083	0.005	0.090	20.5		5.88	1.42
12	6.69	0.02	2.58	0.04	0.250	70.5	0.075	0.004	0.082	18.6		6.81	1.69
13	6.71	0.03	2.27	0.13	0.243	68.6	0.092	0.005	0.102	23.2	0.30	7.62	2.05
14	6.69	0.02	1.88	0.09	0.244	68.8	0.095	0.002	0.108	24.6	1.10	8.61	2.61
15	6.64	0.04	1.53	0.04	0.239	67.4	0.089	0.006	0.104	23.6	1.77	10.19	3.40
16			1.27	0.03	0.235	66.2	0.118	0.003	0.141	32.0	2.74	10.88	4.14
17			1.14	0.05	0.225	63.4					2.81	11.25	4.73
18			1.00	0.09	0.225	63.4					2.23	11.76	5.42
19											2.02	11.24	6.08

* Figures in this column were derived from a previous paper (1).

made on the basis that feathers are composed of approximately 90 per cent protein (keratin). This factor may be neglected in estimating the concentration of the electrolytes.

RESULTS.

The results of the pH determinations were more regular and consistent than we had anticipated (Table I). They showed that be-
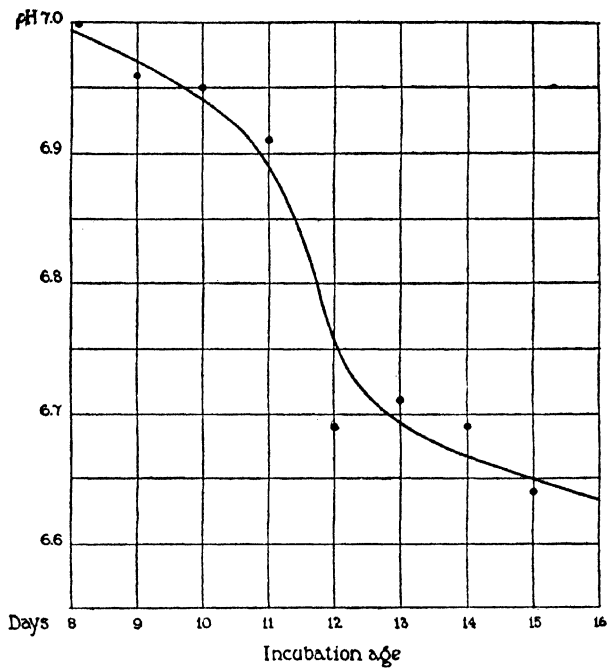
FIG. 3. The pH of the tissues as a function of age.
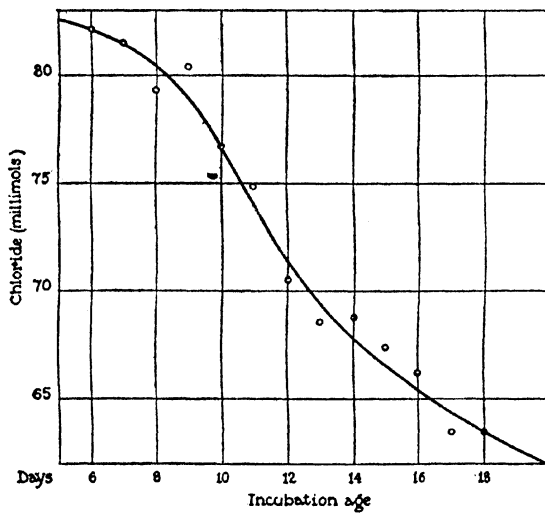
FIG. 4. The molar concentration of chlorides in the tissues as a function of age.

tween the 11th and 12th days of incubation a well marked change towards acidity occurred (Fig. 3).   In form the curve resembles somewhat a simple gelatin titration curve, the steep portion in our diagram indicating a relatively unbuffered phase between two states of comparative stability.

About the same period (Table I) the molar concentration of chloride also falls (Fig. 4) whereas the bicarbonate increases (Fig. 5).   At
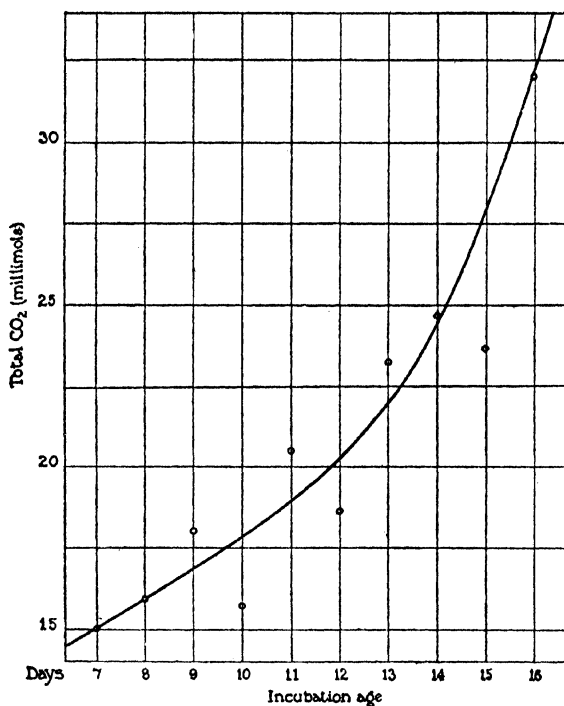
FIG. 5. The molar concentration of total carbonic acid in the tissues as a function of age.

first glance it would seem that this indicated an inverse relationship as if the tendency for maintaining osmotic equilibrium were the controlling factor, but there are reasons for believing that this is not the case.   We assume that the figures for Cl are reliable, and under the conditions of our analyses have significance as indices of tissue conditions.   In the case of carbonic acid, however, certain other

phenomena must be taken into consideration, principally the absorption of carbonates from the shell and their reprecipitation out of solution during active bone formation. Hence the concentration of total acid including as it does the amount of salt in solid phase is no indication of the activity of the ions in functioning protoplasm.

One is inclined to correlate the increasing acidity of the tissues with the accumulation of the CO_2 of catabolism, and to attribute the differences in the rate of pH change to variations in (1) the prevailing ratio in the tissues, (2) the catabolic rate, *i.e.* the rate of CO_2 production, (3) the functional effectiveness of systems of the organism, such as the circulation, which concern themselves with the transport and disposal of carbonic acid, and (4) phenomena, such as bone formation, which affect the carbonate equilibrium.

Little is known about the acid-base ratio in the tissues. From theoretical considerations this balance would seem to be quantitatively the least significant of the factors enumerated; and besides, since the buffer salts of protoplasm are of diverse nature with different dissociation constants, the conditions of heterogeneity would be such as to yield graphically a straight rather than an S-shaped line on titration. Catabolism is also a factor of lesser importance, and since its rate decreases with age it cannot be held accountable for an increasing acidity. Variations in the circulation, on the other hand would greatly affect the pH, and as it has been shown by Cohn (9) that during the early days of incubation there is a marked increase in the rate and regularity of cardiac contraction, and thus presumably a heightened efficiency of the blood vascular system in disposing of the respiratory carbon dioxide, this factor might explain the relative stability of the pH up to the 11th day.

From then on, if one can judge from the heart rate, there being no improved effectiveness in the circulation to compensate for the accumulating CO_2 the hydrogen ion concentration would rise.

The fourth factor listed above, calcium metabolism, may be significantly related to succeeding events. Leaving other facts out of consideration the period of rapidly decreasing pH might be coincidental with the formation of calcium salt deposits at ossification centers which as Little reports (10), may be seen as early as the 9th or 10th day in the chick, whereas the absorption of calcium from the shell,

assuming that this process began about the 12th day, might account for the subsequent relative stability of the pH.

In this connection, Aron's collected analyses (11) of the ash of human embryos indicate that with development there is a relative increase of calcium and phosphorus as compared to potassium, sodium, and choride. The extent of these relative proportional changes may be accounted for by the ossification of the skeletal parts of the body. Aron had no data for carbonates, but on theoretical grounds one might expect a relative increase in their concentration roughly proportional to the phosphates. In the chick Plimmer and Scott (12) have shown that the inorganic phosphates increase markedly during the last days of incubation, being derived from phosphorus organically combined with the lipoids of the yolk. In bone the molar strength of calcium phosphate is to that of calcium carbonate as 3 is to 1. Since our analyses do show an increase in total CO_2, we are led to assume that it is the result of the absorption of carbonates from the shell and their precipitation in osseous structures. For instance:

$$\text{(Shell)} \qquad \text{(Blood)} \qquad \text{(Bone)}$$
$$CaCO_3 + H_2CO_3 \rightarrow Ca(HCO_3)_2 \rightarrow CaCO_3 + H_2CO_3$$

and

$$\text{(Shell)} \qquad\qquad \text{(Blood)} \qquad\qquad \text{(Bone)}$$
$$3\,CaCO_3 + 3\,NaH_2PO_4 \rightarrow 3\,CaHPO_4 + 3\,NaHCO_3 \rightarrow Ca_3\,(PO_4)_2 + 2\,NaH_2PO_4$$
$$+ Na_2HPO_4$$
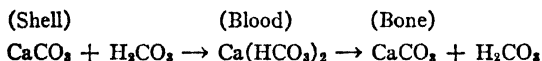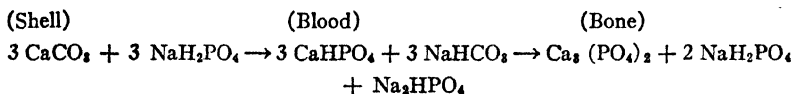
As there is a gradient from left to right, i.e. from shell to bone, it seems that the phenomenon considered by itself would tend to have two significant effects for the present argument: (1) an increase in the alkaline reserve and thus a decrease in the negative acceleration of the pH and (2) the precipitation of calcium carbonate and phosphate as bone, thus increasing the total concentration of these salts in the organism. It is actually the case that soon after the onset of ossification, there is a temporary cessation in the fall of the pH, and an increase in the concentration of carbonate, so that we are inclined at present to account for it on the basis of the initiation of the absorption of calcium carbonate from the shell. The lowered pH would further the absorption of carbonates, a phenomenon, which would

in turn tend to buffer the pH change. If the amount of osseous disposition may be considered as a local affair independent of the general physicochemical equilibrium, how may we ascertain what is the concentration of carbonate in functioning tissue?

For adult blood it has been found that HCO_3 is approximately 0.23 Cl (13) when expressed in mols. This relationship may be fairly universal in protoplasm and body fluids since our figures show that it is approximately true in the case of the tissues of the young embryos before any bone formation occurs. The average values of the concentration of chlorides and carbonates from the 7th to the 10th day inclusive yield the following ratio: HCO_3/Cl equals 0.20. If a constant value for the HCO_3 ratio is assumed, the HCO_3 may be known by a determination of the chlorides as Haldane has suggested (14); furthermore, with a knowledge of the pH one might obtain H_2CO_3 since $pH = 6.1 + \log \dfrac{BHCO_3}{H_2CO_3}$; and, finally, having estimated the total CO_2 by the method described above, the amount of carbonate in bone might be calculated, since:

$$\text{total } CO_2 = H_2CO_3 + BHCO_3 + \text{Bone } CO_3 \text{ (approximate)}$$

In this way theoretical values for the distribution of carbonate in the body, and its concentration in functioning protoplasm might be calculated. The present suggestion that the ratio Cl/HCO_3 has a more or less constant value (*i.e.*, about 0.20–0.23, as found in young chicken embryos and human adult blood) would lead to the conclusion that the concentration of total CO_2 in the tissues decreased with age with the chlorides.

Aron's figures show a decrease in the total Na/Ca ratio no doubt due in large part to bone formation. The concentration of active calcium ions in tissue fluids, assuming a constant supply of calcium, would be functional to the existing physicochemical equilibrium and other factors being equal would tend to increase with the acidity.

This hypothesis might be confirmed or dismissed if it was found possible to make estimations of the amount of bone laid down, or determinations of the CO_2 tension in the tissue fluids or both.

It may be seen (Table I) that the concentration of protein increases markedly with age (Fig. 6), the greatest change occurring around the

15th day—3 or 4 days later than the changes in the concentration of electrolytes.

With the exception of hemoglobin it would seem that the isoelectric points of animal proteins were below pH 6.6, so that with the fall in pH between the 11th and the 12th days the proteins would give up basic ions. Later, with the increase in the concentration of protein, more cations would come to be associated in organic combination; in other words, there would be more protein molecules per gm. of water, though possibly each molecule might be less effective as a
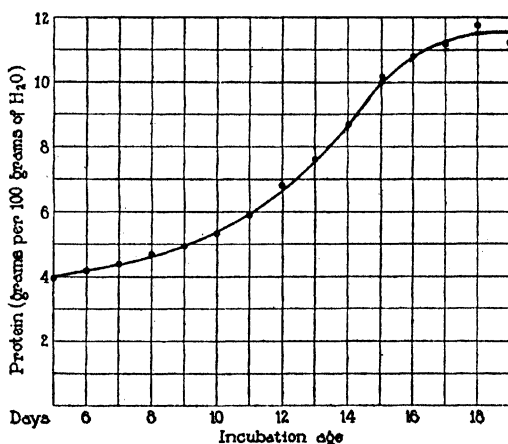
FIG. 6. The concentration of protein (gm. per 100 gm. of water) as a function of age.

buffer. There are no data, however, to show what is the total effect on the protein buffer value made by the changes taking place between the 10th and the 19th days of incubation.

The increase in the proteins, which act as anions at the pH under consideration, serves to replace the deficiency due to the loss of chloride ions. The interesting point is, however, that the two processes are not coincident, but that the chloride concentration has become established at a low level before the concentration of proteins has even reached the point of maximum rate of increase.

In the process of establishing an equilibrium between a mixed salt solution within a collodion sac and distilled water in which it is immersed it is usual to find fluctuations in the relative concentrations of

ions on each side of the membrane respectively due to differences in
permeability or in the rates of migration of ions and molecules, before
stability is attained.   And thus at no moment in the movement
towards equilibrium could the concentrations within and without
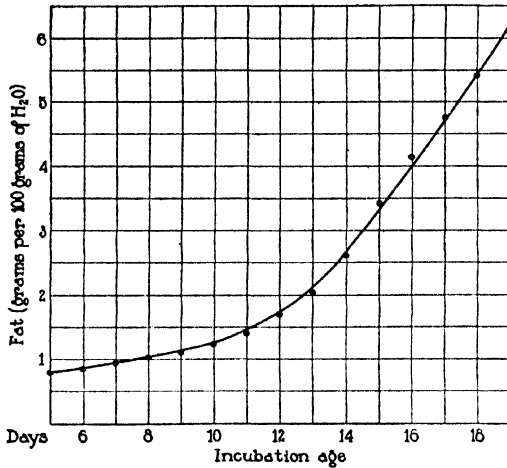
FIG. 7. The concentration of fat (gm. per 100 gm. of water) as a function of age.
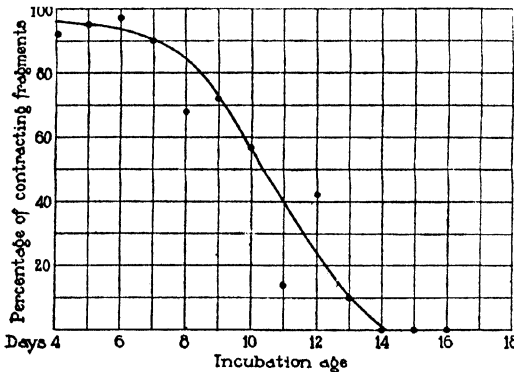
FIG. 8. The percentage of embryonic ventricular muscle fragments auto-
matically contracting after implantation in plasma as a function of age.

the membrane be described by any of the usual physicochemical
equations.

Similarly, results of our observations, which show that the elec-
trolytes probably change several days before the protein, and the

latter several days before the fat (Fig. 7), lead to the conclusion that the processes of chemical differentiation are not to be described by a concept of dynamic equilibrium but rather by a notion of "follow the leader."   The leader in this case is presumably the most rapidly permeating, reactive, and mobile molecule and tentatively we ascribe this rôle to the CO_2 of metabolism.   This subject is to be discussed in a subsequent publication.

It would not be surprising to find that the changes in the chemical constitution of the tissues had considerable physiological significance. Already, in a few cases, we have had occasion to correlate chemical data with functional findings.   For instance, in experiments upon the contraction of ventricular muscle fragments from chick hearts it was found that, whereas in the young embryos almost 100 per cent of the pieces planted in clotted plasma displayed autonomic, rhythmic contractions, in embryos of more than 13 days of incubation age, none contracted spontaneously (Fig. 8).   Combining Cohn's results (15) with our own a graph to represent this phenomenon was obtained.   It may be seen to bear some resemblance to the pH and chloride curves.

<div align="center">CONCLUSIONS.</div>

Investigations of the chicken embryo during its incubation period show that:

1. The pH and the chloride concentration of the tissues decrease with age; the fall is most rapid between the 10th and the 13th days of incubation.

2. The concentration of total CO_2 increases with age.   This fact is not considered inconsistent with a possible decrease in the concentration of active bicarbonate ions, since the increased CO_2 might well be the result of absorption of calcium carbonate from the shell and its precipitation as bone in the embryo.   .

3. The concentration of protein increases with age, especially between the 12th and the 16th days of incubation.

The fact that the electrolytes change with the greatest rapidity at about $11\frac{1}{2}$ days, the protein at 14 days, and the fat at $16\frac{1}{2}$ days might be taken as a demonstration of the phenomenon of unequal development in the realm of biochemical differentiation and consequently

that some notion of order, depending upon molecular reactivity and mobility would describe the process better than any concept of dynamic equilibrium.

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EFFECT OF HIGH PRESSURE ON GERMINATION OF SEEDS (MEDICAGO SATIVA AND MELILOTUS ALBA).*

BY P. A. DAVIES.

(From the Laboratory of General Physiology, Harvard University, Cambridge.)

The application of high pressure (2000 atmospheres) is found to have a definite effect on germination. The action of the high pressure is apparently complex, and seems to involve both physical and chemical changes.

Röntgen (1892) and Tammann (1894) found that the velocity of inversion of cane-sugar by hydrochloric acid increased slightly under 500 atmospheres pressure. Rothmund (1896), Stern (1896), and Cohen and de Boer (1913) observed a result contrary to this; Rothmund observed a decrease of 5 per cent, and Cohen and de Boer of 8 per cent, under 500 atmospheres pressure. Rothmund (1896) and Cohen and Kaiser (1914–15) found a large increase (20 to 37.4 per cent) in the rate of saponification of ethyl acetate by sodium hydroxide under 500 atmospheres pressure. Henderson and Brink (1908) applied 500 atmospheres pressure to frog muscle and found the compressibility was less than 2 per cent. Later, Henderson, Leland, and Means (1908) applied the same pressure to frog muscle for 30 minutes and noted little injurious effect (determined by electrical stimulation). Harvey (1922–23) applied 1000 pounds oxygen pressure and 1800 pounds nitrogen pressure (falling to 1300 pounds during the experiment) for 3 hours to leaves of *Baptisia tinctoria*, without injurious results. Bridgman (1914) applied pressure to egg albumen, and found that pressures below 5000 atmospheres had only slight effect, while higher pressures produced characteristic coagulation of the albumen.

The writer applied 2000 atmospheres pressure[1] at 20°C. to seeds of *Medicago sativa* (alfalfa) and *Melilotus alba* (sweet clover), and found an increase in percentage germination. After the pressures were applied, the seeds not needed for immediate tests were dried on glass plates in a warm room (25°C.) and tested for germination, at 20°C., after 30 days, 6 months, and 10 months.

[1] For the technique of such high pressure experiments, see Bridgman (1914, *a*).

Time of Germination Tests
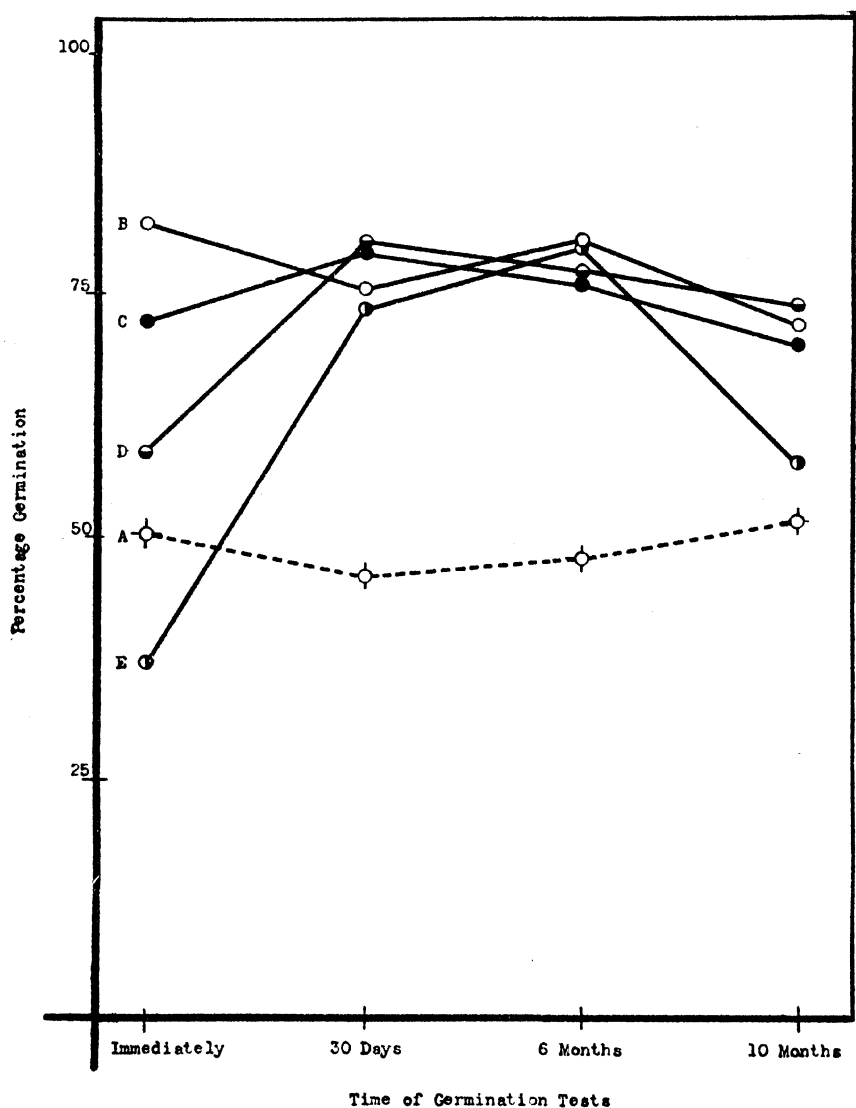
FIG. 1. Showing the effect of pressure on germination of seeds of *Medicago sativa*. Curve *A* (dotted line) represents the percentage germination of the control; *B*, *C*, *D*, and *E*, exposures for 1, 2, 5, and 10 minutes respectively.

Fig. 1 shows the effect of the pressure on germination of *Medicago sativa*. Tested immediately after the pressure was applied, an in-
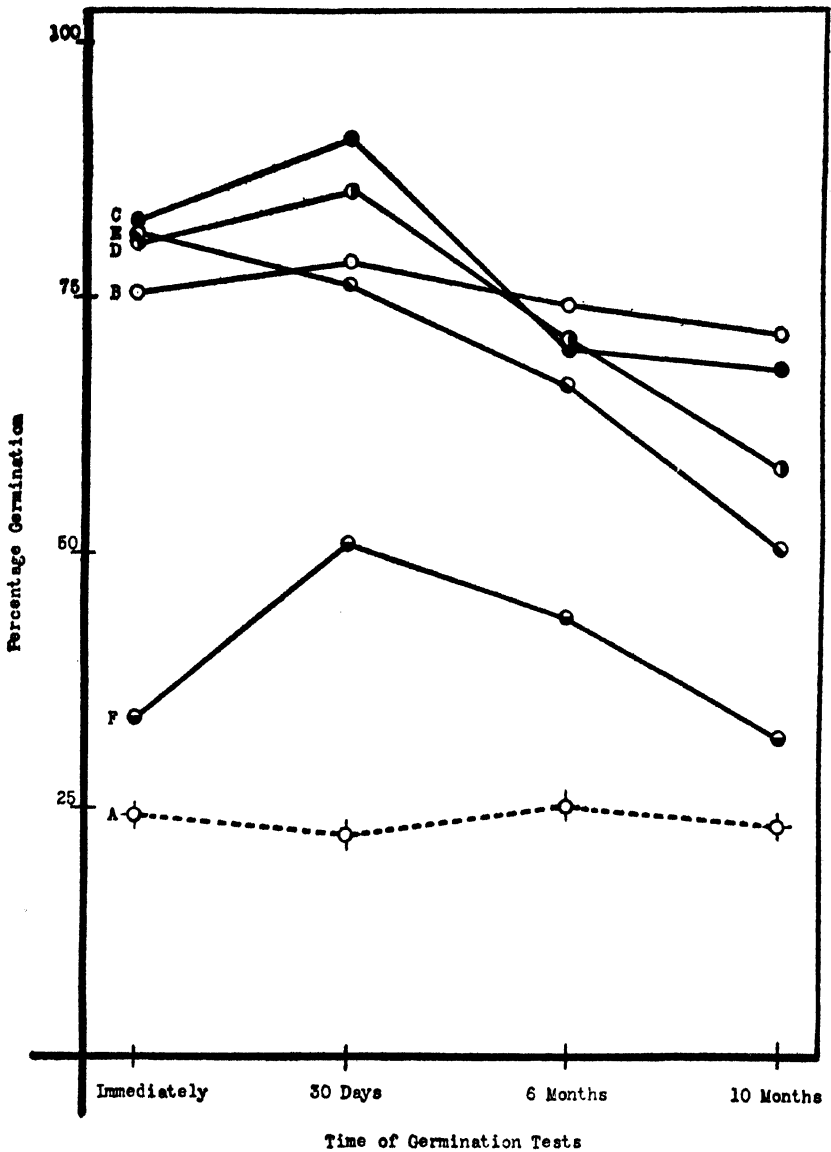
FIG. 2. Showing the effect of pressure on germination of seeds of *Melilotus alba*. Curve *A* (dotted line) represents the percentage germination of the control; *B, C, D, E,* and *F,* exposures of 5, 10, 15, 20, and 30 minutes respectively.

creased percentage germination was obtained from seeds exposed for different lengths of time. *A* represents the control,[2] *B* 1, *C* 2, and *D* 5 minutes exposure respectively. Following 10 minutes exposure (*E*) a decrease in percentage germination is noticed. In the 30 days tests an increased percentage germination is apparent in seeds exposed for 2, 5, and 10 minutes, the increase being greatest in the case of the 10 minutes exposure. The 6 months tests give practically the same results as found for the 30 days tests. In the 10 months tests the percentage germinations for exposures of 1, 2, and 5 minutes were nearly constant, but a rapid drop occurred in the case of the seeds exposed for 10 minutes.

Fig. 2 shows the effect of the pressure on seeds of *Melilotus alba*. In the tests immediately after the pressures were applied, the percentage germinations of the seeds exposed for 5 (*B*), 10 (*C*), 15 (*D*), and 20 (*E*) minutes were practically the same; but with 30 (*F*) minutes exposure, although the percentage germination is above that of the control (*A*), the increase is much less. In the 30 days tests slight increases in percentage germinations are noticed for seeds exposed for 10, 15, and 30 minutes; and in tests after 6 months and 10 months there is a general decrease in percentage germinations, being greater in the tests at 10 months after treatment.

Data later to be published in detail show that 500 atmospheres pressure for longer exposures is less advantageous for germination than shorter exposures at 2000 atmospheres; under low pressures the seeds normally germinating are more rapidly destroyed than the hard impermeable seeds rendered permeable by the pressure treatment. Exposures to 2000 atmospheres at 0°C. give no better results, with respect to germination, than exposures to the same pressure at 20°C.; the only difference being, that at 0°C. longer exposures (approximately $2\frac{1}{2}$ times for seeds of *Medicago sativa* and approximately 5 times for seeds of *Melilotus alba*) are necessary to produce the same increase in percentage germination.

[2] The control is a sample of the original lot from which seeds were taken for pressure experiments, saved, and germinated at the same time (30 days, 6 months, and 10 months) as the treated seeds.

SUMMARY.

An increase in percentage germination is obtained with seeds of *Medicago sativa* exposed for 1 to 10 minutes at 2000 atmospheres hydraulic pressure at 20°C., dried, and germinated after 30 days; and from seeds of *Melilotus alba* under the same conditions of pressure, when exposed for 5 to 30 minutes, dried, and germinated 30 days later. Exposures to 500 atmospheres pressure was less advantageous for germination; the vitality of seeds normally germinating was more rapidly destroyed than the hard impermeable seeds rendered permeable by the pressure treatment. At 0°C., it required approximately $2\frac{1}{2}$ times the exposure to 2000 atmospheres for seeds of *Medicago sativa*, and approximately 5 times the exposure for seeds of *Melilotus alba*, as it did at 20°C.

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BACTERIAL FILTERS.

A Preliminary Note.

By S. P. KRAMER.

(*Cincinnati.*)

We have come to divide bacteria and viruses into filterable and non-filterable, and we have come to think that those organisms, visible or invisible, which are smaller than the pores of our filter are filterable. That size, however, cannot be the sole criterion we have known from the behavior of certain aniline dyes.[1] Thus Victoria blue, a basic dye, will not pass a Berkefeld filter while Congo red, an acid dye, will readily pass through the same filter. Now it happens that the filters which we use in bacteriologic practice, namely, sand, porcelain, and diatomaceous earth, are all of them some form or compound of silicic acid, so that really when one speaks of a filterable organism, dye or other colloid, one ought to say filterable through siliceous filters.

One may speak of a filter when it is in action as a suspension of the material of which the filter is composed, in the fluid which is being filtered. Now silica has a definite negative charge and it may be that if one constructs a filter of a material of charge opposite to that of silica, one might find that bacteria or colloids which are filterable through silica filters are non-filterable through such other filters, and *vice versa*.

Accordingly, filters were made of plaster of Paris and experiments

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[1] In 1916 I showed before the Research Society of Cincinnati that any filter made of siliceous material, sand, porcelain, powdered glass, colloidal silica, or diatomaceous earth would absorb basic dyes, while acid dyes would pass through.

Dr. Stuart Mudd (*Am. J. Physiol.*, 1922–23, lxiii, 429) described the surface of the pores of a Berkefeld filter as the site of an electrical potential difference, a Helmholtz double layer, in which the wall of the filter carried a negative charge and the liquid a positive charge; and predicted and found that when suspensions were filtered through such a filter, positively charged particles were absorbed and retained by the filter.

with various dyes and viruses were made. Thus Victoria blue which does not pass a Berkefeld or siliceous filter readily passes through a filter made of plaster of Paris; while Congo red which readily passes through the Berkefeld filter does not pass through a plaster of Paris filter. If, however, we make a dilute solution of Congo red and render it very slightly acid, thereby changing the color to blue, we find that the blue dye does not pass through the Berkefeld filter but does pass through the plaster of Paris filter. In other words, by reversing the electrical charge one has reversed the filterability.

Experiments have been made with so called filterable micro-organisms and viruses that are available and these experiments are to be continued and extended. One can report now, however, that the bacteriophage of *Staphylococcus aureus* which passes through the Berkefeld filter does not pass through the plaster of Paris filter. The *Vibrio percolans* of Stuart Mudd which passes through the Berkefeld does not pass the gypsum filter. Vaccine virus which passes the Berkefeld filter does not pass the plaster of Paris filter. This same thing is true of rabies virus.

Now when we came to consider the nature of plaster of Paris a very interesting phenomenon was found. Plaster of Paris is supposed to be calcium sulfate but when filters were made of calcined chemically pure calcium sulfate, it was found that such filters had no action on any of the colloid dyes or microorganisms used. Both Victoria blue and Congo red readily passed through such filters. Calcium sulfate is neutral and without charge. Then it was found that the plaster of Paris of commerce contained up to 5 per cent of calcium carbonate, and when calcium carbonate was added to our chemically pure calcium sulfate and filters made from this mixture, such filters acted as did the filters made of commercial plaster of Paris. The calcium carbonate is alkaline and has a positive electrical charge. It is probable that the calcium sulfate in our filters acts as a binder for the calcium carbonate and that it is the calcium carbonate which is the active adsorbing component of our filter.

These experiments were made at the U. S. Hygienic Laboratory at Washington, D. C., and I wish to take this opportunity to convey my thanks for the many courtesies extended by the Director, Doctor G. W. McCoy, and the members of his staff.

ULTRAFILTRATION THROUGH COLLODION MEMBRANES.

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I.

INTRODUCTION.

Since the introduction of the process of ultrafiltration by Martin (1) and the use of collodion membranes (2) as a means of separating dispersed particles from their dispersion medium, much work has been done utilizing collodion sacs in quantitative studies involving the separation of substances in varying degrees of dispersion. Investigators have often contented themselves with merely subjecting the solution under investigation to ultrafiltration through a collodion sac and from the results obtained have drawn conclusions as to the degree of dispersion of the substance under consideration. The effect of the addition of other substances upon the ease with which solutions of colloids pass through a given membrane has been used as evidence of an alteration of the colloid whereas such results may be due to action on the membrane. The passage of a substance through the pores of a membrane is not determined solely by the size of its *invariable* pores, as is often assumed. The mode of filtration, of pressure applied, of the nature of the filter, and of the solution being filtered, may all affect the concentration of any constituent in the ultrafiltrate. These considerations have been neglected in most of the applications of ultrafiltration described in the literature and this neglect explains the wide variation in results recorded by different observers. The conclusions are often quite erroneous and based on doubtful data. This paper aims to present some observations which may help to test the validity of deductions to be made from ultrafiltration experiments.

II.

The Degree of Uniformity in the Sizes of the Pores of Collodion Filters.

The permeability of collodion filters can be varied by varying the concentration of the collodion solution (3); by adding castor oil or glycerol (4); by varying the time allowed for evaporation of the ether and alcohol; by altering the thickness of the sac; or by immersing in aqueous alcohol solutions of varying concentration (5). Despite the fact that sacs obtained by the same technique will show some degree of constancy as regards permeability towards some specific substance (*e.g.* hemoglobin or Congo red), it is still to be expected that some variation in the size of the individual pores should persist despite the most careful manipulations. That this is actually the case is seen in the variation in the amount of water filtered through a series of sacs (Table I). These sacs were all prepared simultaneously and appeared alike in all respects. The same surface was exposed to a pressure of 200 mm. of Hg connected to the sacs arranged in parallel.

TABLE I.

Tube No.	I	II	III	IV	V
Volume filtered in cc. per hour	9.1	9.1	9.9	8.9	9.5

This variation, although not considerable, nevertheless indicates an unavoidable lack of uniformity. Even assuming, moreover, that a series of sacs are prepared so as to appear alike in all respects when tested by their permeability to water, it does not follow that any individual sac will show perfect homogeneity as regards the size of its pores. Indeed, the very nature of their formation seems to delimit this possibility. This fact has usually been overlooked and, as will be seen later, complicates the interpretation of data on the ultra-filtration of many substances. The existence of variation in the size of the pores of a membrane is shown by the ease with which membranes may be prepared which allow a trace of protein or other substance of large molecular size to pass through them. Unless we assume such substances to possess certain particles of a smaller size than the majority, which is not true, then the membrane must possess some pores,

larger than the rest, which allow the passage of the substance in question.

The commonly held belief that collodion holds back only colloidal particles, allowing all crystalloids to pass through, is untrue. No such sharp demarcation exists. The filters ordinarily used in biological work and which are completely impermeable to proteins hold back in part substances of distinctly crystalloidal nature. Thus an eosin solution containing 32 mg. per liter gave an ultrafiltrate containing only 13 mg. per liter, and this, despite the fact that eosin forms a true solution in water (6) as shown by its rapid dialysis (7) and examination under the ultramicroscope (8). Other substances such as sugars (9), non-tans (10), and iso-nitroso-acetyl p-toluol azo-p-toluidin (11) have been described as only partially filterable. And as we shall see later even such simple inorganic salts as sodium chloride and calcium chloride may be partially non-filterable if membranes of sufficiently fine porosity be employed, under certain conditions.

III.

Relation of Pore Size to Dispersion Medium.

It is probable that the actual size of the pores of a membrane is modified by a layer of fluid adsorbed on the surface of these pores, thus diminishing their effective diameter. Thus Tinker (12) in his microscopic study of artificially prepared copper ferrocyanide membranes showed that the particles must be considered as large micellæ around which exists an adsorbed layer of water which diminishes the effective pore diameter of the intermicellar space. Indeed, this view was suggested by Pfeffer (13) as an explanation of the mechanism of semipermeability in his classic experiments on osmotic pressure. The thickness of this adhering layer will naturally be determined by the nature of the medium bathing the pores. The ultrafilterability of a substance will, therefore, be determined in part at least by the nature of the medium in which it is dispersed; and changes in the nature of the medium may alter its ability to pass through a filter.

This view of the nature of the pores of collodion filters seems essential for explaining much that would otherwise seem anomalous. It is in accord, too, with determinations of the actual pore diameters.

Thus the bubble method indicates the size of the pores which allow the passage of collargol to be 200 to 490 $\mu\mu$ whereas filtration through a Chamberland filter would indicate the size of the coarsest particles to be 170 $\mu\mu$ while their actual size is undoubtedly much less (20 $\mu\mu$) (14). The effective pore size is, therefore, much smaller than the actual pore size as determined by the bubble method. The adsorbed film need not behave like the main body of solvent towards any constituent. Thus, the adsorbed layer of water on the copper ferrocyanide micellæ is impermeable to the sugar, giving the membrane its property of semipermeability.

The action of dissolved substances on the thickness of this film may in part at least explain the variations obtained in the rate of passage

TABLE II.

Effect of the Solute on the Rate of Filtration of Aqueous Solutions through Collodion Membranes.

Substance.	Rate of filtration of solution, cc. per hr.	Rate of filtration of distilled water, cc. per hr.
Bile salts (5 per cent)	1	16
Gelatin (1 per cent)	4	15
Strychnine ($\frac{1}{4}$ per cent)	9.1	10.3
NaCl (0.1 M)	11.7	11.4
CaCl$_2$ (0.1 M)	10.2	10.8
AlCl$_3$ (0.1 M)	13.6	15.6

of various aqueous solutions through collodion membranes. A few typical results are given in Table II. The solutions indicated were ultrafiltered under a constant pressure of 200 mm. of mercury and the amount of ultrafiltrate obtained in a given time noted. The rate of ultrafiltration is compared with the rate with which pure water passed through the same filter under the same conditions.

There is perhaps no single mechanism by which the rate of filtration is affected and hence no individual physical constant can be used in evaluating this effect. Electrolytes might conceivably affect the rate by altering the charge of the membrane double layer (15). Viscosity changes would affect the rate in accord with Poiseuille's law (16). The proteins most probably act by forming an adsorbed layer

on the wall of the pores which diminishes their size. The action of surface active materials such as bile salts is especially marked and seems to depend on their ability to alter the conditions of the layer adsorbed on the walls of the pores. Examples of these three types of substances are given in Table II.

On the basis of the above theory of a variable effective pore size dependent on the medium in contact with it, one can explain many of the changes in permeability on the addition of otherwise inert materials. Thus many dyes, *e.g.* tetrachlorophenolsulphonphthalein, sodium carminate, etc., appear in the ultrafiltrate in higher concentration from Ringer's solutions than from distilled water. The increase in permeability described by Brinkman and von Szent-Györgyi (17) may be explained in the same way although attempts to render the collodion sacs used in this investigation permeable to hemoglobin by the use of bile salts, sodium oleinate, lanthanum chloride, acid or alkali, etc., failed. They always remained impermeable to this substance.

IV.

The Effect of the Filtering Pressure on the Concentration of the Ultrafiltrate.

If collodion filters, as has been demonstrated above, contain pores of various sizes, the nature of the ultrafiltrate will be influenced by the pressure used in forcing the solution through the filter. This problem has been discussed by McBain and Jenkins (18).

Theoretically, a pressure above the osmotic pressure of those substances which are retained by the filter should be necessary to cause the passage of the remaining substances through the filter. If, *e.g.*, a solution, containing a single solute A, whose osmotic pressure is p, be ultrafiltered through a filter containing a series of graded pores, some of which permit the passage of A while others do not, the concentration of A in the ultrafiltrate will depend on the pressure employed in the ultrafiltration. If a pressure less than p be employed, separation of the solvent through the pores through which A cannot pass should not occur, and hence the solution should pass unchanged through the larger pores, giving an ultrafiltrate of the same composition as the original solution. As long, therefore, as the filtering pres-

sure is less than the osmotic pressure of the retained constituents, none of these can be separated from the solvent. The use of such pressures should result in the production of an ultrafiltrate in which the diffusible solute is present in the same concentration as in the original solution. To test this hypothesis, solutions were ultrafiltered at pressures less than their calculated osmotic pressures. These substances failed, however, to appear unchanged in concentration in the ultrafiltrate. Instead, with decreasing pressures there was a gradual increase in the concentration of the ultrafiltrate. The explanation of these results seems obvious when we consider the actual conditions prevailing during filtration. Those pores through which a constituent cannot pass are bathed, more or less, by the ultrafiltrate outside of the membrane. The effective pressure necessary to cause separation of pure solvent will, therefore, be the difference in osmotic pressure of this constituent in the solution and ultrafiltrate. Consequently, even at pressures much below the osmotic pressure of a constituent which cannot pass a pore, separation of solvent will occur with consequent dilution of the ultrafiltrate.

The degree of this dilution of the ultrafiltrate will depend on the pressure employed. The quantity of fluid, Q, passing through a tube of length, L, and diameter D in time, T, under a pressure, p, is given by Poiseuille's formula:

$$Q = \frac{K\, D^4\, p\, T}{L}$$

From this equation in which K is a constant, one would expect that a change in pressure would not influence the concentration of the ultrafiltrate since the relative changes in the amount of solution passing through large and small pores should be equally affected. However, since this simple relationship does not apply to the pores of the magnitude of collodion membranes, this condition will not obtain, and the concentration of ultrafiltrate will vary continuously with pressure as may be seen in the following typical example (Table III).

This fact complicates the process of ultrafiltrations and makes it impossible to take advantage of McBain and Jenkin's suggestion; *i.e.*, to work below some definite pressure. This pressure will vary continuously and even under pressures much lower than the osmotic

pressure of the substance filtered, dilution of the ultrafiltrate will occur. In general, however, the lower the pressure, the closer does the concentration of ultrafiltrate approximate the true solution and hence results obtained at low pressures are most accurate.

The results of Table III show the great variations in the concentration of the ultrafiltrate which a change in pressure produces. If a large quantity of solution be added to a filter and the filtration continued, without further addition of new solution, there will be a gradual increase in the concentration of the residue left in the filter. This increased concentration of the residue will produce a gradual increase

TABLE III.

Substance.	Concentration of solution ultrafiltered, mg. per 100 gm. of water.	Pressure, mm. of mercury.	Concentration of ultra-filtrate, mg. per 100 gm. of water.
Rose bengal.	1000	50	850
"	1000	20	940
"	1000	10	970
"	5	30	3.5
"	3.2	200	1.5
"	3.2	2	2.5
Eosin.	1000	2	1000
"	1000	25	980
"	1000	50	850
"	1000	100	840
"	1000	700	800
"	3.2	200	1.8
"	3.2	10	2.3

in the concentration of the ultrafiltrate as well. Hence consistent results are only obtainable if the same condition of filtration and the same degree of concentration of the residue are attained in successive experiments.

This problem of the influence of pressure on the concentration of the ultrafiltrate through collodion filters has been usually neglected and reference to the literature shows many instances where such influence, although demonstrated, has been either ignored or misinterpreted. Thus Burian (19) found sodium chloride to appear in lesser concentration (about 10 per cent) in the ultrafiltrate than in the original serum or protein solutions when filtered under a pressure of 10

atmospheres. Similar results were likewise obtained by Reid (20). These results are explicable on the assumption that there are pores whose effective size is modified by a layer of adsorbed solvent and which, in these experiments, could not allow the passage of the sodium chloride, but at the high pressures used could transmit the solvent. With increasing pressure these smaller pores with their anomalous filtering power enter into greater consideration.

TABLE IV.

Results of the Ultrafiltration of Solution of CaCl₂ Containing 80 Mg. Ca per 100 Gm. H₂O through Protein-Treated Collodion Membranes under 250 Mm. Hg Pressure.

Tube No.	Concentration of ultrafiltrate, mg. per 100 cc.	Concentration of residue, mg. per 100 cc.
1	78	83
2	78	82
3	79	81

TABLE V.

Concentration of the Residue Left after Ultrafiltering 30 Cc. of a CaCl₂ Solution Containing 80 Mg. of Ca per 100 Gm. of H₂O to a Final Volume of 10 Cc.

Tube No.	Concentration of residue, mg. per 100 cc.
1	113
2	95
2	95

The production of an ultrafiltrate of too low composition by the use of high pressures is demonstrated by the results of Table IV.

By using a higher pressure—1 atmosphere—more striking results were obtained as shown in Table V. These results may in part explain the low and discordant values obtained in ultrafiltration studies of the Ca in blood (21). The use of membranes differing as regards the size and uniformity of their pores and the use of varying pressures must result in such discrepancies.

IV.

The Effect of Adsorption by the Membrane on Ultrafiltration.

An insidious error to be guarded against in the use of collodion membranes to obtain quantitative measurements of the state of a substance in solution, is a failure to obtain a true ultrafiltrate due to adsorption of the substance under investigation by the filter (22). One must ensure that a real filtration is taking place and that the results are not vitiated by adsorption.

The adsorption by the filter of substances to which it is impermeable is likewise of great importance in modifying the nature of the ultrafiltrate. This is especially true of solutions containing proteins such as blood plasma or other tissue fluids which have been widely used in biological work. The adsorption of a film of protein on collodion filters was described by Loeb (23) and has been quantitatively studied by Hitchcock (24). A filter on whose pores a layer of protein has been adsorbed will obviously be less permeable than before treatment. This would be especially marked if we consider the more hydrophilic nature of the protein as compared to collodion, and the consequently increased thickness of the layer of adsorbed solvent.

The manner in which this phenomenon may lead to misinterpretation of results is exemplified by the recent work of Rosenthal (25). As a result of ultrafiltration studies of rose bengal in the presence of proteins, he concluded that this substance is completely bound since none of the dye appears in the ultrafiltrate under such conditions. The impossibility of such a conclusion is, of course, obvious when one considers the nature of the adsorption of dye on protein which necessitates the existence of a considerable concentration of the former in the free condition at equilibrium. As a matter of fact, dye enters the collodion sac and is found in the ultrafiltrate after an initial adsorption of some dye by the filter. This ultrafiltrate cannot be considered as the true concentration of free dye since only a fraction of the dye comes through from an aqueous solution, which, we have already seen, is due to impermeability of some of the pores to this substance. This fact would preclude the possibility of using collodion as a means of determining the binding of this dye. Furthermore, clogging of the pores by the protein helps to still further diminish the actual amount

obtained in the filtrate. The protein test suggested by Rosenthal, and based on a supposedly complete binding of rose bengal, is due to the buffering action of the protein and would be given by any buffering solution.

The use of collodion for determining the degree of peptization of colloids is likewise open to objection on the same grounds. The fact that a fixed and constant percentage appears in the ultra-filtrate does not necessarily indicate that this fraction is in a state of greater dispersion than that contained in the residuum. Nor would an alteration of this filterable quantity on the addition of acid neces-sarily indicate the true peptization since the addition of the latter in itself might affect the composition of the ultrafiltrate. The same criticism is applicable to other studies based on the partial permeability of the filter (26).

The effect of adding surface-active materials such as sodium lysal-binate, the bile salts, etc., and the increase in permeability consequent on such additions should be noted. Their action may result from an effect on the adsorption by the membrane and not on the degree of dispersion of the colloid under investigation as has been assumed.

V.

Comparison of Ultrafiltration and Dialysis as a Means of Determining the Binding of Phenol Red by Blood Albumin.

In a previous paper (27) the adsorption of phenol red (phenol-sulfonphthalein) by proteins has been studied using ultrafiltration as a means of obtaining the intermicellar fluid; and the results thus ob-tained were applied in studying the excretion of this substance by the kidney (28). The nature of phenol red is such as to permit its study in this way. Its highly crystalloidal character which manifests itself in the extreme rapidity with which it diffuses through membranes such as parchment, enables it to pass through collodion in unchanged concentration even when the pores of the latter are at their minimum size. Moreover, the small extent of its adsorption by collodion makes practical its use for such studies.

That ultrafiltration can be used to study the binding of certain sub-stances is shown by the following experiments in which the results of

ultrafiltration are compared with those obtained by dialysis where such factors as difference in pore size, adsorption of protein with consequent clogging of pores, etc., would not vitiate the results.

The binding of a 4 per cent albumin solution was determined in the following manner. The solutions containing phenol red were placed in diffusion shells (Schleicher and Schüll No. 579) which were closed by a rubber stopper and placed in a glass tube but slightly larger than the shells, in order to permit the use of a minimal quantity of pure water in the outer tube. The dye quickly diffuses into the outer fluid and equilibrium was insured by shaking the tubes until the con-
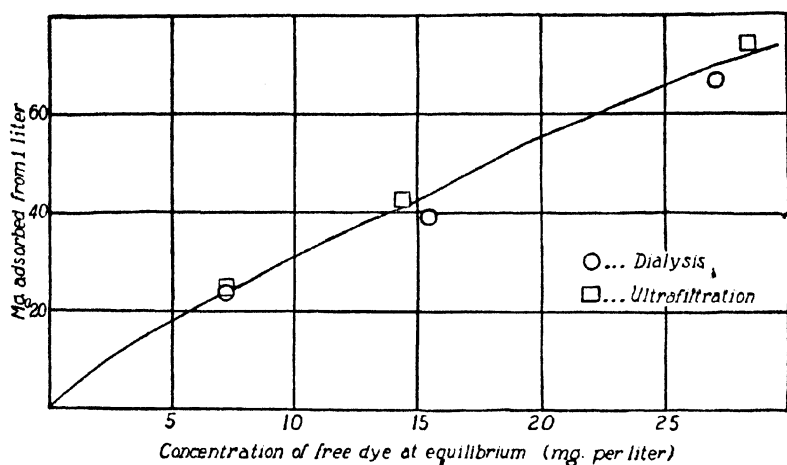
FIG. 1. Comparison of ultrafiltration and dialysis for determining the binding of phenol red by blood albumin.

centration of dye in the outside fluid became constant (50 hours). None or negligible amounts of protein were found in the outer fluid. From such determinations the amount of dye bound was determined and could be compared with that previously found by ultrafiltration. The agreement which is within experimental error is shown in Fig. 1.

This agreement indicates that filtration of albumin solutions through the filters under the pressures and conditions used in the investigations described for phenol red (27) are valid as indicating a true separation of intermicellar fluid. At higher pressures or using denser membranes high values would undoubtedly be obtained. The application of the

same method to other dyes or crystalloids towards which collodion shows a lesser degree of permeability is, however, subject to considerable error.

<div align="center">VI.</div>

The Effect of Variations in the Method of Preparation of the Membranes.

It will be clear from the above consideration of the effect of the size of the pores, pressure employed, etc., that the nature of the ultra-filtrate may be altered by variations in the structure of the membranes employed. In some cases such variations will be more pronounced than others depending on the relation of the size of the constituents in the ultrafiltrate to the size of the pores of the membrane. Standardization of the method of preparation is difficult since slight variations in the concentrations of the collodion used, the thickness of the membranes, duration of the time allowed for drying, etc., all affect the physical properties of a membrane, including the pore size. Each experimenter has used his own technique in the preparation of his membranes and in the method of filtration and consequently the wide divergence of the results recorded in the literature is what would be expected from the conditions outlined above. Even using the same technique, one obtains slight variations as shown in Tables I and IV. In Table V is shown the divergence possible as a result of varying the thickness of the membrane. Tube 2 of this table was the same as those employed in obtaining the data of Table IV. In preparing Tube 1, attempts were made to obtain an exceedingly fine porosity and the results of this variation in the method of preparation manifests itself in the divergence of its value from those obtained with the tubes used in the other experiments.

Unless the same details of procedure are followed as were used in making the collodion sacs used in this investigation, the results cannot be duplicated. Thus filtration of calcium chloride solutions through more porous membranes would show the same concentrations in the ultrafiltrate and solution ultrafiltered. This would be no safe criterion, however, for the use of blood serum or protein solutions as the latter might cause a sufficient decrease in the size of the pores to invalidate their use in determining the state of calcium in the solution

studied. Use of membranes of a finer structure, on the other hand, might give results for the binding of phenol red much higher than the true values as obtained by dialysis experiments.

The membranes used in this laboratory are made in the following manner. An 8 per cent solution of dry negative cotton in a mixture of equal parts of absolute alcohol and ether serves as the source of the collodion. This solution is poured into seamless test-tubes (18 mm. in diameter), rotated for several minutes to give a uniform coating to the glass and allowed to dry (mouth of tube projecting downwards) for 30 minutes. A second coating is then similarly applied and after the excess ether has evaporated (about 30 minutes) the membrane is removed and preserved in distilled water in the ice chest. These membranes are perhaps the most suitable for filtrations of biological material at low pressures. The use of high pressures on comparatively thick and dense membranes is likely to involve greater errors.

VII.

CONCLUSIONS.

It is obvious that the factors considered in this paper render data obtained by ultrafiltration open to criticism unless they are checked by other methods and precautions are taken for the elimination of the vitiating effects which have been described.

As regards the mechanism of ultrafiltration, the view of a sieve-like action as most experimental evidence indicates, is adequate, if all the factors are considered which might modify the effective pore size. The behaviors of collodion membranes which seem contrary to a mechanism of ultrafiltration based on the existence of a system of pores, can be explained on the basis of a variable layer of adsorbed fluid on the walls of the pores. It is, therefore, unsound to make any deductions about living tissues from the demonstration of changes produced in the behavior of collodion membranes. Thus, the increase in the rate of filtration of water through collodion by diuretics (29) or the change of permeability due to the presence of surface-active materials, gives us no information about their action in the living organism. The effect of these substances on a sieve-like membrane of the type of collodion would not necessarily bear any analogy to that

exerted on the emulsion type of membrane of living cells. The mechanisms of the reactions necessary to produce the same effects in such widely differing systems may be entirely unrelated.

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A CONTRIBUTION TO THE THEORY OF PHAGOCYTOSIS.

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In endeavours to reduce the phenomenon of phagocytosis to a physical basis, account has been taken of three principal factors. The first of these relates to the chance contact of cell and particle, which has been admirably dealt with by McKendrick from a theoretical point of view (1), and by Fenn from an experimental aspect (2). The second factor controlling the ingestion is that of the viscosity of the cell; the importance of this has been much emphasised by Loeb (3), although theoretical investigations are still to be made. The third factor is the nature of the surface conditions between cell and surrounding fluid, particle and surrounding fluid, and cell and particle, the consideration of which has led to information of much value, but unfortunately to a certain amount of controversy, the conclusions of Rhumbler (4) having been amplified by Tait (5), whose statement of the problem has been criticised by Fenn (6) and by Mudd and Mudd (7).

In this paper, we shall venture to add a note relating to this controversy, and thereafter to consider in outline a fourth factor, hitherto, so far as we are aware, unmentioned—the influence of electric charge of cell and particle on the phagocytosis.

1. The Surface Forces.

If we have a system composed of cell, rigid particle, and surrounding fluid, in which there are three interfacial tensions—that between the particle and the fluid, S_1, that between the cell and the fluid, S_2, and that between the particle and the cell, S_{12},—then when the system is in equilibrium, there must be a contact angle θ between cell and particle, which satisfies

$$\cos \theta = \frac{S_1 - S_{12}}{S_2}.$$

If there is equilibrium at the contact angle, then, from the nature of the action of surface forces, the whole system must be at equilibrium, and if so, the free energy is at a minimum.   Further, θ must be single-valued, and the expression does not refer only to spheres, an important point, for phagocyted particles are not in general spheres.

The limits of the left-hand member of the equation are (-1) and $(+1)$.   The limits of the right-hand member are $(-\infty)$ and $(+\infty)$. There will accordingly be equilibrium at partial ingestion with a real value of θ when the value of the right-hand member falls within the limits of the left-hand member; if the value falls outside these limits, there will be no real angle of contact, and incomplete ingestion cannot satisfy the equilibrium.   The condition for spreading of the cell on a rigid surface is the same as that for ingestion.   We have $\cos \theta = (S_1 - S_{12})/S_2$, and if the value of the right-hand member gives a real value of θ, to do which it must lie between (-1) and $(+1)$, incomplete spreading will satisfy the equilibrium conditions; if the value falls outside these limits, either no spreading at all, or spreading to infinity, will result.   This follows simply from the fundamental expression when the particle is rigid and when the surface upon which spreading takes place is rigid; their curvatures, moreover, are not involved; if we have to treat the ingestion of a non-rigid particle, or spreading on a non-rigid surface, there is a little more difficulty. These cases do not concern us.

There are thus five possibilities.   The value of $(S_1 - S_{12})/S_2$ may be

(a) less than (-1).   This condition is not mentioned by Tait or by Fenn; it is possible, and the particle would flow on the cell.   In the case of a rigid particle, no ingestion would result.

(b) equal to (-1).   The particle will not be ingested, but will be in equilibrium at the cell surface.

(c) greater than (-1) and less than $(+1)$.   In this case, θ has a real value, and there will be equilibrium at incomplete ingestion. Tait ignores this case, for which Fenn rightly criticises him.   Fenn has shown by an admirable computation of a numerical example that such a condition results in minimum free energy at incomplete ingestion of the particle; we confess, however, without in any way wishing to underrate this excellent computation, that the result appears to us to

follow directly from the fundamental equation, and to require no laborious proof. Apart from this, there is the question as to the frequency of the occurrence of this condition in actual practice. For pure liquids, for example, it never arises, and whether it is of frequent occurrence in systems such as we are considering remains to be shown. Fenn's appeal to experiment, in pointing out that incomplete spreading, which is associated with the same conditions as incomplete ingestion, does in fact occur and can be easily recognised, is not sufficient to settle the question, as incomplete spreading may be brought about by the operation of factors unconsidered in these equations. An apparent equilibrium may result, for instance, because the time allowed for spreading is not indefinitely great. It may thus well be that Tait has ignored a condition which does not occur in practice, or which is rare; had he been dealing with the engulfing of a drop of a pure liquid by another drop, we take it that he would have fallen into the same error, but he would have been, in fact, correct nevertheless, for the case omitted does not occur.

(*d*) equal to (+1). This is a special case of (*e*).

(*e*) greater than (+1). This is Tait's condition for complete ingestion, which is correct so far as it goes, although we note that Tait derives it by putting $\theta = 0$. This would give (*d*); (*e*) is given, not by $\theta = 0$, but by unreal values of θ, associated with positive impossible values of $\cos \theta$. This is the common, indeed invariable, case with pure liquids, and implies both complete ingestion of the particle by the cell, and also spreading to infinity by the cell, as Fenn points out.

Fenn's statement of the case is therefore perfectly correct, and his criticism of Tait justifiable. Tait's statement is correct as far as it goes, but omits all cases except (*d*) and (*e*). We add this note for two reasons, apart from recording our agreement with Fenn. First, we think it should be recognised that because case (*c*) is a mathematical possibility, it is not necessarily a common occurrence. Secondly, we feel that the conditions for spreading, incomplete ingestion, complete ingestion, etc., follow somewhat more simply from the initial conditions than has been indicated.

2. *The Electrical Forces.*

It is generally admitted that in many suspending fluids both the cells and the particles which undergo ingestion have a like charge. We have to consider how this charge affects phagocytosis.

The charge on a particle is generally looked upon as being due to a double layer at the particle boundary. The origin of the double layer is a matter of some doubt, but we propose to take it to be produced as follows. Owing to molecular configuration, the surface of the particle presents a series of point charges of one sign, say positive. Oriented by these is an equivalent number of charges of opposite sign at the surface of the surrounding fluid, while a second layer of positive charges, deeper in the surface of the fluid, surround this first layer. It will be understood that the particle is really surrounded by a kind of atmosphere made up of many layers, but that this division into two only is the result of a species of summation in the inner and outer parts of the atmosphere. The whole system is in kinetic equilibrium, and in the layers at least, if not on the surface of the particle, redistribution of charge is possible.

For the purposes of this problem, although we should hesitate to extend the idea in its simple form to other cases, the particle may be regarded as a solid sphere, bearing a charge, which may or may not be distributable, and surrounded by a spherical shell, in which distribution is possible. The charges on the surface of the sphere are in equilibrium with those on the inner surface of the shell; the charges on the outside of the shell are in equilibrium with all external space. Moreover, since these equilibria exist, the charge on the particle together with that on the inside of the shell may be removed entirely from the system, and do not affect in any way the distribution on the exterior of the shell, which is influenced only by the presence of neighbouring conductors in external space.

If the particle be regarded as such a system, then the cell bearing a like charge will be a similar system, and the influence of the one on the other will be on the distribution of charge on the external surface of the shells, and will in no way involve the internal surfaces or the surfaces of cell or particle, which may be regarded as being removed from the system.

Suppose a shell B, corresponding to the particle of radius b, is very small compared with a, the radius of a shell A, representing the cell. Let A have potential V and charge E, while B has charge e. If the centre of A is O, and that of B is P, the effect of e on the distribution on A may be taken as being given very nearly by the single image at P'. Further, the induced density on B due to the distribution on A is very nearly that due to a charge kV/a at O, and $-ea/c$ at P^1, c being the distance from O to P. These vary with the cube of the distance of an element of B from O or P', and so the distribution on B may be taken as uniform.

The charge and potential on B are thus

$$e,$$

$$e\left(\frac{1}{b} - \frac{a}{c^2 - a^2}\right)k + V^2\frac{a}{c},$$

and that on A

$$E\ (=\ kVa - ea/c),$$

$$V.$$

The energy of the whole system is thus

$$\mathbf{E} = \frac{1}{2k}e^2\left(\frac{1}{b} - \frac{a}{c^2 - a^2}\right) + \frac{V^2\,ka}{2},$$

$$= \frac{1}{2k}\left(\frac{E^2}{a} + \frac{2Ee}{c}\right) + \frac{1}{2k}e^2\left(\frac{1}{b} - \frac{a^3}{c^2\,(c^2 - a^2)}\right).$$

If we separate the shells by a distance dc, $\mathbf{E}$ will alter by $d\mathbf{E}/dc \cdot dc$, and $-d\mathbf{E}/dc$ is the repulsive force between the spheres in the line of centres. Now

$$-k\cdot d\mathbf{E}/dc = \frac{Ee}{c^2} - e^2\,a^3\,\frac{2c^2 - a^2}{c^3\,(c^2 - a^2)^2}$$

or

$$kVe\,\frac{a}{c^2} - e^2\,\frac{ac}{(c^2 - a^2)^2}.$$

According as this expression is negative, zero, or positive, the force is an attraction, zero, or a repulsion. If c is only a little greater than a,

that is, if the small shell is very near to the surface of the large one, $(c^2 - a^2)$ is very small, and the force is an attraction. More specifically, if E and e are of the same sign, there will be an attraction when

$$E < ea^3 \frac{2c^2 - a^2}{c\,(c^2 - a^2)^2}$$

while if this expression is an equality, from which

$$kV/e = c^3/(c^2 - a^2)^2,$$

there will be no force between the shells at all. Such will be an equilibrium position, but an unstable one.

Returning to the idea of the cell and the particle, both of the same sign, when these are at a distance, there will be a repulsive force between them; as the particle is moved up to the cell, this force becomes at first greater. It thereafter lessens, and, when the particle is near to the cell, vanishes. The particle passes through this point of unstable equilibrium, and is now attracted to the cell, until the two come into contact. At this moment the charges redistribute once more, and equalise. A removal of the particle to a very small distance from the cell surface reestablishes the attraction, and to remove the particle to the position of unstable equilibrium, work has to be done. There is thus a force, manifested as soon as the particle leaves the cell surface, which tends to draw it back towards the surface, and prevents its withdrawal into space. The particle will therefore tend to remain at or within the surface, and to be subject to the action of such surface forces as prevail. By the *surface* must, of course, be understood the surface of the hypothetical shell.

The action of these forces may be used to explain the frequently observed fact that the ingestion of a particle is preceded by a phase when the particle appears to be stuck to the surface of the cell. Fenn remarks that this preliminary stage is clearly a surface tension phenomenon, by which we take him to mean that it is a very early stage of ingestion, for he points out that it is due to the fact that the surface of the cell exposed to the plasma is decreased. We suggest, however, that the first stage is that the particle is attracted to the cell surface, and that the electrical conditions are such as will prevent its removal; hence it remains. Thereafter the early stage of ingestion takes place,

followed by more and more complete ingestion, until an equilibrium is reached.

This treatment may be objected to upon two grounds at least, quite apart from any criticism directed against the special conception of the Helmholtz double layer. The first objection may be that the values of E and e are small in the case of cells suspended in a fluid, and that therefore the resulting induction and forces will be small. This is true, but these small forces appear sufficiently large to produce very important effects—such as the stabilising of cell suspension and of bacterial suspensions—and so ought to be taken into account unless there is excellent reason for assuming that their effect is negligible. The second objection may be that the above treatment applies only if b, the radius of the particle, is very small compared with a, the radius of the cell. This is also true, and is a fundamental assumption; if the particle and the cell approximate to one another in size, a different treatment, in which not one, but an infinite series of images, are inserted, is required, and a different result is forthcoming, the attractive forces being scarcely manifest, if at all. This may account, or play a part in accounting, for the greater ease of ingestion of small particles than of large—a phenomenon remarked upon by Tait and also by Fenn. As we intend later to use the fuller electrostatic treatment in connection with another subject, we shall leave the matter as it stands for the present, content with pointing out that the above treatment applies only if the particle is very small compared with the cell.

In conclusion, it should be pointed out that the introduction of these electrostatic forces in no way affects previous work on chance contact, for now we require, not the contact of the particle with the cell surface, but its approach to the position of unstable equilibrium. The chance of the one happening in a mixture of cells and particles is governed by the same conditions as the chance of the other. Nor have the electrostatic forces any influence on the surface tension conditions as laid down by Fenn. Their only effect is to tend to retain the particle at the cell surface, and thus to render it under the influence of the surface forces for a longer period of time. In this way they may tend to reduce the importance of the viscosity factor, for, if the particle were in contact with the cell surface for only a very short time, the viscosity of the cell, determining the rate at which the cell could flow over the

particle, would be exceedingly important, whereas, if the particle were to remain at the surface, a slower flow of the cell substance would result in ingestion.

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THE PHYSIOLOGICAL PRINCIPLE' OF MINIMUM WORK APPLIED TO THE ANGLE OF BRANCHING OF ARTERIES.

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In a recent paper[1] it has been proposed that the total work involved in the circulation of blood in a section of artery (sufficiently small so that the pulsating changes in the kinetic energy of the blood stream in it can be neglected as compared to the work required to overcome friction) can be expressed by the equation:

$$E = pf + bvol = \frac{f^2 \cdot l \cdot 8\eta}{\pi r^4} + bl\pi r^2 \tag{1}$$

which embodies Poiseuille's law of flow and a term which covers the cost of maintenance of blood volume. p is the fall in pressure in dynes/cm.², b is the cost of blood volume in ergs/cc. sec. (considered constant), vol is the volume, r is the radius of the section of artery, and η is the viscosity of whole blood (also taken as "constant"). At constant flow, f (that is. for any given steady state), and at constant length of arterial section, l, the total energy, E, is a minimum when:

$$f^2 = \frac{r^6 \pi^2 b}{16\eta} \tag{2}$$

Substituting for f^2 in the original equation, we obtain:

$$E/l = r^2(3\pi b) \tag{3}$$

Or, to avoid constants, we may use a different unit for E, and write:

$$(kE)/l = r^2 \tag{4}$$

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[1] Murray, C. D., *Proc. Nat. Acad. Sc.*, 1926, xii, 207.

This equation is a simple deduction from premises discussed at length in the paper referred to.[1] This equation can be used to develop a theoretical law for the angle of branching of arteries, on the assumption that the total work of the circulation is to be a minimum, that is, assuming the validity of equation (4). Let us consider the plan of arterial branching which is most efficient in allowing for the distribution of blood from a point S to two points, A and B (see Fig. 1). r_0, r_1, r_2 are the radii of the arteries; l_0, l_1, l_2, the lengths, and x and y
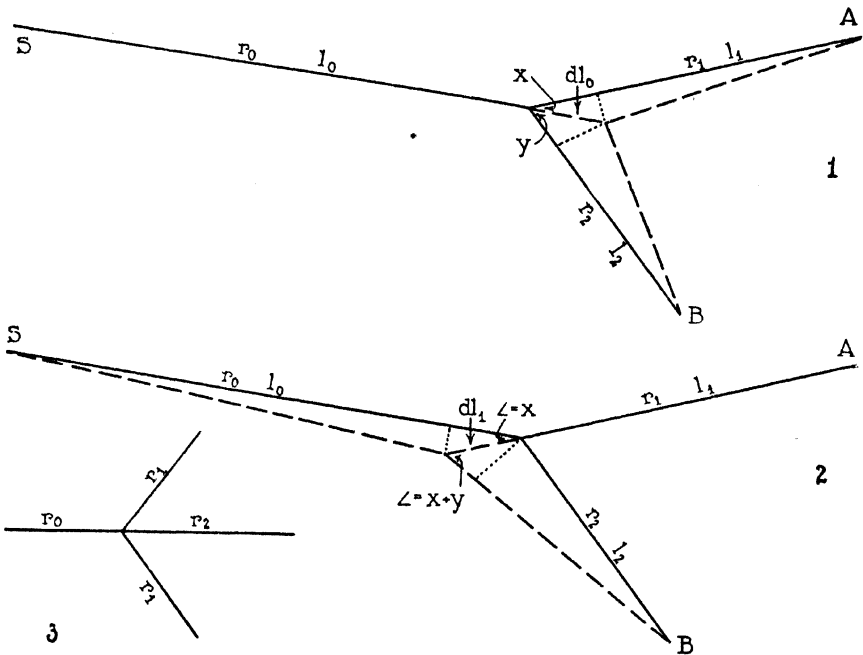
Fig. 1.

are the angles, to be determined, which the branches make with the line of direction of the main artery proceeding from S. Suppose Diagram 1 to represent the condition for minimum work, and then imagine an infinitesimal increment, dl_0, to be added to l_0 (l_1 and l_2 now assuming the positions indicated by the dashed lines). The "cost" (in the tentative unit of kE) of the section l_0 is now increased by the increment $dl_0 r_0^2$, and the costs of the branches are decreased (in this case) by $\cos x \, dl_0 r_1^2$ and $\cos y \, dl_0 r_2^2$ respectively. The dotted lines

show the triangles constructed in order to arrive at this result. Two similar constructions (one of them illustrated by Diagram 2, Fig. 1) can be made, representing virtual increments added in turn to l_1 and l_2. By the principle of virtual work in mechanics (which states that, when conditions are such that the total work is a minimum, then a virtual change in the configuration of the system results in no change in the total work) we obtain one equation for each of the three constructions, as follows: (Only two of these equations are independent, since cos $(x+y)$ is a function of cos x and cos y, but the procedure is simplified by taking the three cases.)

$$dl_0 r_0^2 = \cos x \, dl_0 r_1^2 + \cos y \, dl_0 r_2^2$$
$$dl_1 r_1^2 = -\cos(x+y) \, dl_1 r_2^2 + \cos x \, dl_1 r_0^2$$
$$dl_2 r_2^2 = -\cos(x+y) \, dl_2 r_1^2 + \cos y \, dl_2 r_0^2$$

These equations can be divided through by dl_0, dl_1, and dl_2 respectively, and by combining we arrive at the equations:

$$\cos x = \frac{r_0^4 + r_1^4 - r_2^4}{2 \, r_0^2 \, r_1^2}; \quad \cos y = \frac{r_0^4 + r_2^4 - r_1^4}{2 \, r_0^2 \, r_2^2}; \quad \cos (x + y) = \frac{r_0^4 - r_1^4 - r_2^4}{2 \, r_1^2 \, r_2^2}$$

Since the flow in the branches must equal the flow in the main stem, and since, from equation (2), at maximum efficiency $f = k'r^3$, then it follows that $f_0 = f_1 + f_2 = k'r_0^3 = k'(r_1^3 + r_2^3)$. Thus $r_0^3 = r_1^3 + r_2^3$. Substituting this last relation in the equations for the angles we obtain the final result:

$$\cos x = \frac{r_0^4 + r_1^4 - (r_0^3 - r_1^3)^{\frac{4}{3}}}{2 \, r_0^2 \, r_1^2}; \quad \cos y = \frac{r_0^4 + r_2^4 - (r_0^3 - r_2^3)^{\frac{4}{3}}}{2 \, r_0^2 \, r_2^2}; \quad \text{or,}$$

$$\cos (x + y) = \frac{(r_1^3 + r_2^3)^{\frac{4}{3}} - r_1^4 - r_2^4}{2 \, r_1^2 \, r_2^2} \qquad (5, 6, 7)$$

two of the three equations being sufficient to determine the plan.

If, instead of dividing into two branches, an artery, after giving off two *equal* branches on opposite sides, continues in its course, the ideal angle made by either branch with the main artery can be determined according to the following equations (see Diagram 3, Fig. 1):

$$dl_0 r_0^2 = dl_0 r_2^2 + 2 \cos x \, dl_0 r_1^2$$

$$\cos x = \frac{r_0^2 - r_2^2}{2 \, r_1^2} = \frac{r_0^2 - (r_0^3 - 2 \, r_1^3)^{\frac{2}{3}}}{2 \, r_1^2} \qquad (8)$$

A few scale drawings are presented illustrating the application of the above equations. The equations yield results conforming to certain qualitative laws observed by Roux and quoted in a paper by Hess:[2] for example the rule that the larger the branch the more the
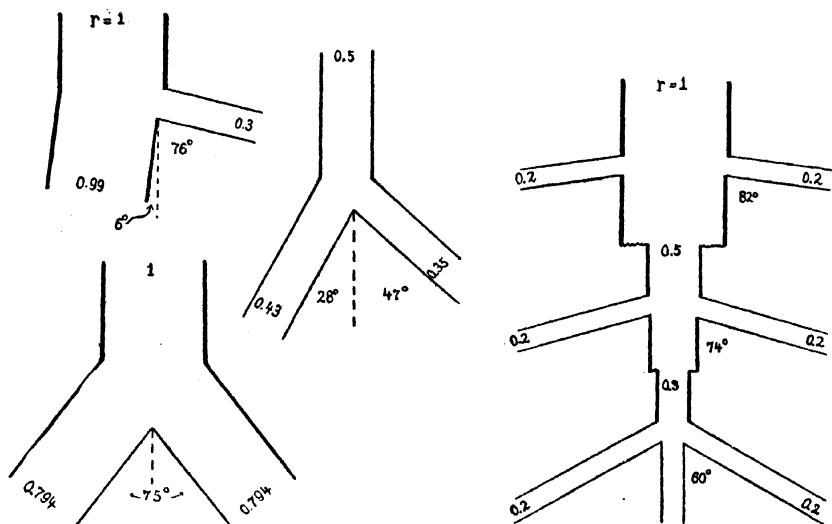
FIG. 2. Scale drawings of arterial branching. The drawings are based on the equations developed in the text, and illustrate the conditions, for free unobstructed branching of vessels, which make the work of distribution of blood a minimum. Values for the radii of the vessels and for certain angles are shown in the drawing. The sum of the cubes of the radii of the branches equals the cube of the radius of the main stem from which they arise. The confluence of veins appears to follow the same rules in a general way.

It is interesting to compare these branchings with branching in trees. (The drawing should be turned upside down for this comparison.) For blood vessels the minimum theoretical angle for a simple bifurcation is 75°, but in trees the actual angle is usually less than this, especially when the bifurcation occurs in a vertical plane and may therefore be supposed to be affected by both helio- and geotropic factors. Another interesting comparison is afforded by results obtained by Miss E. Hendee and Miss M. E. Gardiner in this laboratory. If a tree is cut through at any point of the trunk, of a branch, or of a small twig, the ratio of the weight of the whole part (peripheral to the cut section) to the cube of the radius of the cut section (taking the average ratio observed for each set of branches of similar weight) is a constant. Thus the rule for arteries, $r_0^3 = r_1^3 + r_2^3 + \ldots$, tends to hold also in *small* trees.

[2] Hess, W. R., *Arch. Entwcklngsmechn. Organ.*, 1903, xvi, 632.
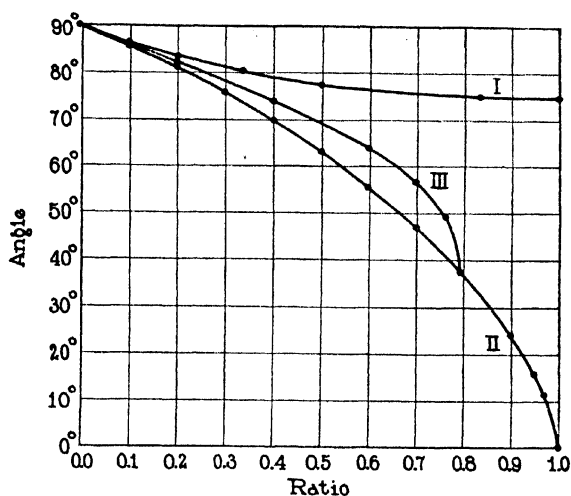
FIG. 3. Curve I shows the relation between the angle $(x+y)$ and the ratio r_1/r_2—the case illustrated by Diagram 1. The curve passes through a minimum when $r_1 = r_2$. See Table I. Note the small variation in the angle of bifurcation, whatever the value of the ratio.

Curve II shows the relation between angle x and the ratio r_1/r_0 (Table II, Diagram 1). Note that the larger the branch the less is the angle made with the line of direction of the main stem.

Curve III shows the relation between angle x and the ratio r_1/r_0 (Table III, Diagram 3). This is the only case considered where the direction of the main artery is unchanged. At the point when the ratio equals 0.7937, this case merges into the one illustrated by Curve II, the continuation of the main stem having been reduced to zero.

TABLE I.		TABLE II.		TABLE III.	
See Diagram 1.		See Diagram 1.		See Diagram 3.	
r_1/r_2	$\angle (x + y)$	r_1/r_0 or r_2/r_0	$\angle x$ or $\angle y$	r_1/r_0	$\angle x$
1	74°.95	(0.00)	90°.0	(0.00)	90°.0
2 or 1/2	77°.6	0.20	81°.2	0.20	82°.3
3 " 1/3	80°.3	0.40	69°.9	0.40	74°.2
5 " 1/5	83°.5	0.60	55°.6	0.60	64°.1
10 " 1/10	86°.5	0.80	36°.7	0.70	56°.7
(∞ " 0.0)	90°.0	0.90	23°.9	0.76	49°.2
		0.95	15°.5	(0.7937)	37°.5
		(1.00)	0°.0		

main artery is deflected, and the smaller is the angle between the branch and the direction of the main stem before division; and the rule that branches of equal size make equal angles with the same main stem.   Anatomically the angles are the variables which can be checked most readily, and it is not necessary here to consider the lengths, which are, in each case, easily determined, if the points have been given and the angles calculated.

An equation for the "ideal" angle for a single branching has been published by Hess, who took as his criterion of efficiency the condition which would make the fall in pressure, between a point in the main artery and a point in the branch, a minimum.   He neglected to take into account the conditions in the continuing portion of the main artery, assuming implicitly also that the continuation was in a straight line.   Owing to this error Feldman[3] has been somehow misled.   He arrives at the result[4] that, as the ratio of the radius of a branch to the radius of the artery approaches unity, the angle of bifurcation should approach zero.   He then cites as an instance the small angle between the external and internal carotid arteries.   In contrast to this curious result, the rule arrived at in the present paper is that the angle in the bifurcation of an artery should not be less than 75° (74°.9 to be more exact).   Miss M. Hardy and Miss M. S. Gardiner, in this laboratory, have prepared a corrosion model of the arterial and venous systems of a cat's lung,—an excellent organ in which to observe free branching of vessels.   Examination of this preparation makes it apparent that for every bifurcation angle less than let us say 70° there are hundreds, if not thousands, of cases where the angle is between 75° and 90°, a fact consistent with the limits for the angle $(x+y)$ as given by equation (7).   These limits are perhaps the most characteristic results of our analysis.   There occur, of course, numerous and important branchings which do not agree exactly with the simple theory, e.g. the bifurcation of the pulmonary artery makes too wide an angle, and the common iliac arteries make an angle varying from 60°–75° (Piersol) and therefore preponderantly on the narrow side of the theoretical angle.   It would be interesting, in the case of the carotids, to analyze factors of

[3] Feldman, W. M., Biomathematics, London, 1923.
[4] Feldman,[3] p. 172.

differential growth which make this case, and presumably others, singularly exceptional. One more point: in analyzing the minimum properties of physiological systems it seems preferable, when possible, to construct the problem in such terms that the *work* involved in the system becomes a central feature.

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